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ORIGINAL ARTICLE

Effect of metronidazole supplemented with hydroquinone on the adhesion of *Lactobacillus acidophilus* in ovine vaginal cells

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Abstract This work demonstrates that the addition of metronidazole together with a ubiquitous quinone compound reduces adherence of *Lactobacillus acidophilus* to ovine vaginal cells.

Spectrophotometric and voltammetric studies have shown that neoformed compounds were observed in these systems; there were also changes in their electroactive composition, and the oxidant status had a significantly higher value compared to the control ($p < 0.05$). Based on reduction potential (E; mV), the distribution of electroactive compound concentrations suggests that the compounds with low reduction potential induce this behavior, which would indicate that the addition of metronidazole with a ubiquitous quinone compound to the vaginal system might increase the reductive capacity of these systems.

This work shows that the study of behavior and fluctuations of the redox compounds that compose the vaginal environment, in terms of concentration and species of redox molecules, must be hierarchized in order to better understand the early stages of colonization by microorganisms.

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PALABRAS CLAVE
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Células vaginales;
Ovinos;
Redox;
Voltametría

Efecto del metronidazol suplementado con hidroquinona en la adherencia de *Lactobacillus acidophilus* en células vaginales ovinas

Resumen Este trabajo demuestra que la incorporación de metronidazol conjuntamente con un compuesto quinónico ubicuo disminuye la adherencia de *Lactobacillus acidophilus* a células vaginales ovinas.

Los estudios espectrofotométricos y voltamétricos mostraron que en estos sistemas aparecieron compuestos neoformados y que hubo modificaciones en la composición electroactiva; asimismo, el estatus oxidante presentó un valor significativamente superior con respecto al control ($p < 0,05$). Según los potenciales de reducción (E; mV), la distribución de las concentraciones de los compuestos electroactivos muestra que los compuestos con bajos potenciales de reducción inducen este comportamiento. Esto indicaría que la incorporación de esta mezcla al sistema vaginal aumentaría su capacidad reductora.

El trabajo muestra que el estudio del comportamiento y las fluctuaciones de los compuestos redox que componen el ambiente vaginal, en términos de concentración y especies moleculares, debe ser jerarquizado para comprender mejor las primeras etapas de la colonización de este ambiente por parte de los microorganismos.

Introduction

Microbial adhesion to surfaces is the first step of the early processes not only in beneficial but also in pathogen microorganism installation in many diverse ecological niches[24]. At this first stage of bacterial adherence processes there are physicochemical conditioners acting in non-specific interactions (such as electrostatic repulsion and Van Der Waals' force)[23,24]. Moreover, it has been shown that in cell–cell adhesion processes there is intervention of molecules that can undergo intra- and inter-molecular interactions with molecules or ubiquitous functional groups, such as H_2O_2 or thiols, through chemical reactions as well as redox reactions[5,9,20]. Based on these facts it is evident that the factors affecting these physicochemical variables must be especially considered in order to characterize this biological scenario[8,13]. Studies on bacterial adherence in bovine vaginal cells showed that the presence of oxidant compounds, such as periodate ions, may affect this process[11], which suggests that the redox status of an extracellular environment, defined in terms of intensity (reduction potential; Eh) and capacity (number of electroactive compounds)[14], might be a significant proximity factor[13] in microorganism adherence to the vaginal mucosa.

Metronidazole (MTZ) (Fig. 1a) is an antibiotic used in the digestive, reproductive and skin systems. The mechanism of action consists in inhibiting the synthesis of nucleic acids. MTZ and other nitroimidazole derivatives are active redox compounds which perform a significant antibiotic activity based on intracellular reduction from a nitro group to a nitro radical ($R{-}NO_2 + e^- \rightarrow R{-}NO_2^{\bullet -}$)[2], which is the reason why they are widely used in various microbial ecosystems inside the animal's body, including the vaginal environment[10,19].

Quinones are molecules that play important roles in living organisms, such as the e^- transfer in photosynthesis and in vitamin K. They are mostly benzoquinones, naphthoquinones and anthraquinones. Their important feature for this work is their ability to act reversibly in redox processes. Currently, natural and synthetic quinone derivatives are being strongly studied because of their chemotherapeutic activity against diseases such as cancer; furthermore, they have antimicrobial, anti-inflammatory and antioxidant properties[1,21].

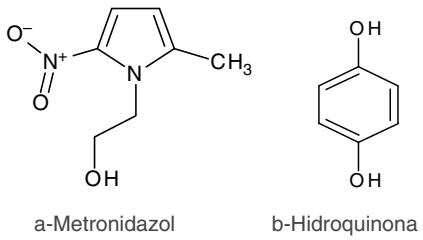

Figure 1 (a) Molecular structure of Metronidazol; (b) molecular structure of Hidroquinona.

Independently of its antibiotic nature, when MTZ is in the vaginal-mucus-physicochemical-environment, it may act as a redox effector interacting with other ubiquitous redox compounds such as quinines (Fig. 1b), and in biological processes that are affected by redox conditions, for example in microbial adherence to vaginal cells.

This work aimed to assess the effect of adding MTZ alone and MTZ with hydroquinone (H_2Q) (as a model of ubiquitous redox compounds in biological systems) on *Lactobacillus acidophilus* adherence to ovine vaginal cells suspended in simulated vaginal fluid, in order to hierarchize the study of the electrochemical scenario as a systemic view.

Materials and methods

Ovine vaginal cells

Ovine vaginal epithelial cells (VECs) were taken with a sterile swab from the vaginal cavity of experimental animals.

This technique consists in separating the lips of the vulva, introducing the sterile swab into the dorsal commissure bypassing the clitoral fossa in dorsocraneal direction and rotating it in order to extract the cells.

Samples were put inside tubes containing 10 ml Minimum Essential Medium Eagle (MEM) (Sigma®). After agitating the mixture for 2 min with a Vortex agitator, the swab was removed and VECs remained refrigerated (4 °C) until they were used. Before carrying out the trials, cells in the MEM medium were centrifuged at 200 *g* for 10 min. The sediment was resuspended in sterile saline solution (SS) and washed twice (200 *g*; 10 min) to eliminate native bacteria[11]. Finally, cells were resuspended in 3 ml synthetic vaginal fluid (SVF) to give a final concentration of 10^4 cells/ml (Thoma counting chamber).

Sterile swabs were used to obtain ovine vaginal fluid from the experimental animals (the same technique described to obtain the cells was used). Swabs were put in 4 ml SS, agitated in Vortex, and filtered with 0.2 μm (Millipore®). Synthetic vaginal fluid was also used, and it was prepared using the formula suggested by Owen and Katz[12] modified (glucose: 10 g/l; glycerol: 0.16 g/l; lactic acid: 2 g/l; acetic acid: 1 g/l; urea: 0.5 g/l; NaCl: 5.5 g/l; KOH: 1.4 g/l CaOH: 0.22 g/l; yeast extract: 3 g/l; pH: 6)[4].

The strain used in this study was *L. acidophilus* ATCC 314 (USA), which was kept in suspension with skimmed milk at −20 °C until it was used. Bacteria were activated in modified LAPT broth (proteose peptone: 2%; yeast extract: 1%, glucose: 1% Tween 80: 0.1%; pH: 6.5) at 37 °C for 24 h with two picks every 12 h.

Bacteria were harvested by centrifugation at 3500 × *g* for 15 min at room temperature. Sedimented cells were washed twice with SS and resuspended in sufficient solution to give a final concentration of 10^7 cells ml/l (Thoma counting chamber), corresponding to 10^6 UFC/ml (MRS agar). This bacterial suspension was used for the adherence assays, named bacteria.

Adherence assays

In order to incubate VECs with *L. acidophilus* in various treatments, 0.5 ml of bacteria was added to 0.5 ml VEC suspension. The systems were incubated during 1 h at 37 °C. After incubation, the treatments were centrifuged at 70 × *g* for 10 min, then washed in accordance with the protocol suggested by Prats et al.[16], and were resuspended in SS. Following the incubation period, aliquots of the resulting suspension were stained with Tinción 15 (Biopur®), then placed on microscope slides, and cells and bacteria were counted under the microscope (Axiolab Zeiss Jena Gm).

The MTZ solution (0.125 ml; 0.14 mmol/l) and H2Q solution (0.125 ml; 0.01 mol/l) were used for the experiments.

The treatments were: (i) bacteria and cells (Control), (ii) bacteria and cells supplemented with MTZ, (iii) bacteria and cells supplemented with H_2Q, (iv) bacteria and cells supplemented with MTZ and H_2Q. All the experiments were carried out in triplicate.

The results were expressed as an adhesion percentage (%), defined as the number of VECs with adhered bacteria, divided by the total number of VECs observed and multiplied by 100[11].

Molecular fractionation

Molecular fractionation of ovine vaginal fluid suspensions was done by putting 0.4 ml aliquots in a matrix of Sephadex G25® gel (Pharmacia). Gel calibration was carried out with Blue Dextran (MW: 2.106 Da; CAS No. 115-39-9) and Bromophenol Blue (MW: 669.97 Da; CAS No. 87915-38-6) following Cooper's technique (1984)[3]. Using a fraction collector (Roucaire, Retriever II, France), 26 fractions were recovered (2.8 ml each), which were measured for absorbance at 200 nm (Hitachi U, 1500 France).

Voltammetric measurements

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were used to characterize the electroactive components in the studied systems. A POL 150 (Radiometer Analytical, France)[17] equipment was used to record current/potential scans. The experiments were carried out with no oxygen after purging the system with O_2-free N_2 for 3 min before performing each voltammetric scan. In every case, the supporting electrolyte used was KCl 0.04 mol/l. The working electrode was a vitreous carbon electrode; the reference electrode was a Pt foil, and every potential was measured in relation to a calomel standard electrode (CSE). Each sample underwent five repeated voltammetric scans until a reproducible cycle was reached, and the average for this value was found using the Trace-Master 5 software (Radiometer Analytical). CV scans started in the negative direction at +0.700 V up to −1.900 V with 0.025 V/s scan speed.

In order to study the electrochemical changes in the systems of VECs and bacteria supplemented with H_2Q and MTZ, DPV scans were performed. Scans were performed between +0.500 V and −1.300 V (step duration: 0.4 s; width: 5 mV). The voltammetric cell contained 900 μl of analyte (constituted by bacteria in SS (0.5 ml; 10^7 cells/ml), the VEC suspension in SVF (0.5 ml), 0.125 ml of 10^{-2} mol/l H_2Q and 0.125 ml of 1.5×10^{-4} mol/l MTZ), 2000 μl of dimethyl sulfoxide (DMSO), 2000 μl O_2 – free distilled H_2O and 100 μl of 2 mol/l KCl as supporting electrolyte. In every case, final MTZ and H_2Q concentrations in the voltammetric cell were, respectively, 2.5×10^{-6} mol/l and 1.7×10^{-4} mol/l. In DPV, the current unit/potential unit (nA/mV) quotient was recorded for each reduction potential value. These values are linearly dependent on element concentration (Radiometer, 2002) and were used to determine the system-oxidant-status, following the expression used by Pidello[14], defining the variable by adding the reduction potential (intensity factor) (E; Volts) multiplied by the electroactive compound concentration (capacity factor) in the various redox pairs in a given system:

$$\text{oxidant status} = \sum_{i=1}^{n(par)} E_i(\text{mV}) \times [\text{oxidated species; nA/mV}]$$

MTZ, H_2Q, DMSO and KCl compounds were proanalysis reactive and were used without additional purification.

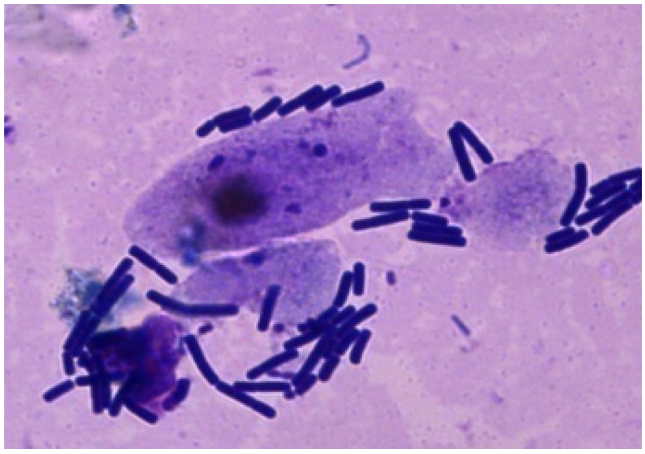

Figure 2 Adhesion of *Lactobacillus acidophilus* ATCC 314 to ovine vaginal epithelial cells suspended in synthetic vaginal fluid (SVF).

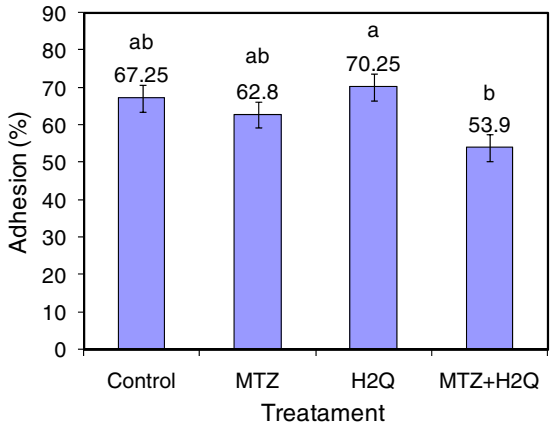

Figure 3 Adhesion (%) of *Lactobacillus acidophilus* ATCC 314 to ovine epithelial cells in systems treated with metronidazole (MTZ), hydroquinone (H2Q) and metronidazole plus hydroquinone. Similar letters indicate that there are no statistical significant differences ($p < 0.05$).

Statistical analysis

The Chi-squared distribution by means of the test of independence was used to analyze frequency in bacterial adherence studies. The *t*-Student test[15] was used to evaluate the differences between treatments in the voltammetric studies.

Results

Adhesion of *L. acidophilus* ATCC 314 to ovine vaginal cells

Figure 2 shows adhesion of bacterial cells in the studied system. Adhesion studies were conducted with aliquots of VEC suspension and *L. acidophilus* cells both unsupplemented and supplemented with MTZ, H_2Q or MTZ and H_2Q. Figure 3 shows the results of adding *L. acidophillus* to ovine vaginal cells, expressed as an adherence percentage as described in Materials and Methods.

The results show that adhesion of *L. acidophillus* ATCC 314 to ovine VECs with H_2Q was significantly affected by the supplementation with MTZ plus H_2Q ($p < 0.0001$). In the latter case, adhesion decreased significantly. Moreover, differences were found between Control and systems supplemented with MTZ or with H_2Q alone ($p > 0.05$).

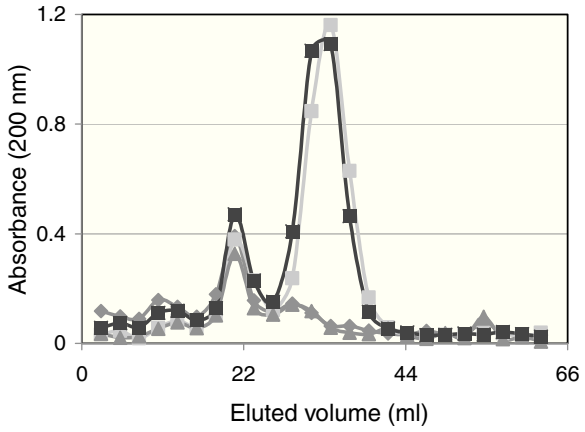

Figure 4 Spectrophotometric behavior (200 nm) of fractions obtained after elution of ovine vaginal fluid suspensions alone (-◊-), supplemented with metronidazole (-△-), supplemented with hydroquinone (-□-), and supplemented with metronidazole plus hydroquinone (-x-), in Sephadex G25® gel.

Spectrophotometric behavior of the studied suspensions

Figure 4 shows the spectrophotometric behavior of eluted solutions of unsupplemented and supplemented ovine vaginal fluid after fractionation with Sephadex G25. Absorbance values, measured at 200 nm, indicate that the fraction with greater molecular weight (excluded fraction) in the ovine vaginal fluid samples was only slightly increased when it was supplemented with H_2Q or MTZ (13%). The absorbance values of the solution containing H_2Q (0.1 l; 0.01 mol/l) had a very well-defined signal at 200 nm, which was observed in the fractions eluted with 30–40 ml, with an approximate 1.2 absorbance value, coinciding with the behavior observed in previous studies[25].

MTZ molecules (0.14 mmol/l; 0.1 ml) were eluted with 20–30 ml, and these values coincided with the elution volume of ovine-vaginal-fluid-components, which showed a signal at wavelength 200 nm. Although the MTZ absorbance signal under these fractionation conditions linearly responds to higher concentration (data not shown), with the amounts used in these experiments this signal was not different from the absorbance of ovine vaginal fluid alone (Fig. 3).

Voltammetric behavior

MTZ and H_2Q aqueous solutions

Figure 5 shows the result of CV scans performed between +0.700 and −1.900 V of the MTZ at a 4×10^{-3} mol/l final concentration with deoxygenated distilled water or with

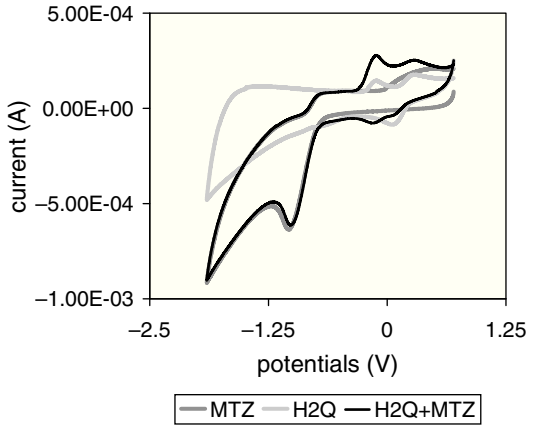

Figure 5 Cyclic voltammetry (CV) scans of metronidazole (MTZ), hydroquinone (H2Q), and metronidazole plus hydroquinone solutions. Every potential was measured against the CSE. Voltammograms started from a negative direction at +0.700 V with 0.025 V s^{-1} scan speed. For further details see Materials and Methods.

an H_2Q 7×10^{-4} mol/l aqueous solution, and H_2Q aqueous solutions (7×10^{-4} mol/l). The solution containing MTZ alone had a single reduction peak (−1.034 V) and its corresponding oxidation peak (−0.758 V), with $E_{1/2} = -0.890$ V and $\Delta E_p = 0.276$ and, therefore, electron transfer was irreversible under these conditions. The solution containing H_2Q alone showed a single peak in the cathodic wave (+0.086 V), whereas in the anodic wave there were two peaks corresponding to oxidation occurring in two stages of one electron each (−0.103 V and +0.275 V), which is characteristic of quinone behavior in unbuffered aqueous systems[18]. When two compounds constituted the same solution, MTZ did not change its electroactive behavior, while H_2Q increased the intensity of anodic peaks and showed two signals corresponding to complementary cathodic peaks.

Ovine vaginal cell and *L. acidophilus* ATCC 314 suspensions

Based on the adherence percentages shown in ''Adhesion of *L. acidophilus* ATCC 314 to ovine vaginal cells'' section and the results observed in ''MTZ and H_2Q aqueous solutions'' section, in order to study the electrochemical characterization of systems, only the systems supplemented with H_2Q, and MTZ plus H_2Q were retained. DPV scans indicate that these systems showed a total concentration of electroactive compounds (nA/mV) of 158.2 ± 4.7 and 166.7 ± 11.2 respectively, and these values did not differ statistically if compared between each other ($p < 0.05$). However, when analyzed by potential reduction ranges, the treatments differed significantly at various potential values ($p < 0.05$). While the systems supplemented with H_2Q alone had greater electroactive compound concentrations in the −0.2/−0.4 V and −0.6/−0.8 V ranges, the systems supplemented with MTZ and H_2Q had greater concentrations in the 0.4/0.2 V and −1.0/−1.2 V ranges (Fig. 6). The calculation of oxidant status, including only the potential ranges which differed significantly, showed -40.2 ± 1.5 and -44.3 ± 1.1 values for the treatments supplemented with H_2Q or MTZ plus H_2Q respectively, which also differed significantly when they were compared ($p < 0.05$) (Fig. 6, inserted figure).

Discussion

Given the fact that the redox status is a conditioner of bacterial adherence, in this work we studied systems where: (i) there was an intention to modify adherence by adding an imidazole molecule (MTZ), which is a potential effector of the redox status due to its chemicals structure, and (ii) there was also an intention to modify speciation of this molecule by adding another redox effector (H_2Q).

Under certain conditions and concentrations, MTZ inhibits *Lactobacillus* spp. growth (from 1000 μg/ml), while under other conditions it stimulates growth (between 128 and 256 μg/ml)[22]. In this work, 25 μg/100 ml of MTZ were employed, which would be innocuous for the strain used[22]. This fact was confirmed in this work, showing that adherence does not differ significantly when the control and the system supplemented with MTZ are compared. Furthermore, it was observed that adherence is not different in the control compared to the system supplemented with the quinone compound used (H_2Q) to modify MTZ electrochemical behavior. However, the results showed that *L. acidophillus* ATTC 314 adherence to ovine VECs changed by the presence of the MTZ and H_2Q combination. This result suggests that the chemical composition and redox status[20] of the system were modified.

The spectrophotometric characterization of the various mixtures (Fig. 4) supports this interpretation, as it shows that when MTZ and H_2Q molecules constituted the same solution there is a slight increase in the fractions with greater molecular size after fractionation with Sephadex G25. In addition, as the absorbance scan was performed at 200 nm, the neoformed compounds eluting at this fraction were unsaturated and had a greater molecular mass than H_2Q.

Voltammetric scanning (VS) of pure compound aqueous solutions (Fig. 5) provided a clear signal of MTZ with $E_{1/2}$ value of −0.890 V, which is therefore moved toward negative potentials in relation to the values presented in the bibliography[6,7]. Zuman and Rupp[26] explain that these movements, which occur both in aqueous means and in solutions with different percentages of organic solvents, are due to pH increase and to the existence of an acid–base balance that precedes the transfer of the first e^- of the R–NO_2 group to form the nitro radical. This interpretation, which suggests a possible neoformation of electroactive species, might explain the results obtained in an aqueous means, which is a potential proton donor. On the other hand, CV scans also show that the presence of both MTZ and H_2Q changes the electroactive compound concentration in potentials ranging between −0.500 and +0.500 V, indicating that each mixture has a characteristic oxidant status, which helps anticipate that, as these mixtures are redox effectors, they generate significantly different electroactive behaviors in the systems where they are included[20].

After performing DPV scans, when the electroactive behavior in the H_2Q solution and the H_2Q and MTZ mixture in the presence of ovine cells and bacteria was grouped by potential ranges (Fig. 6), significant differences between

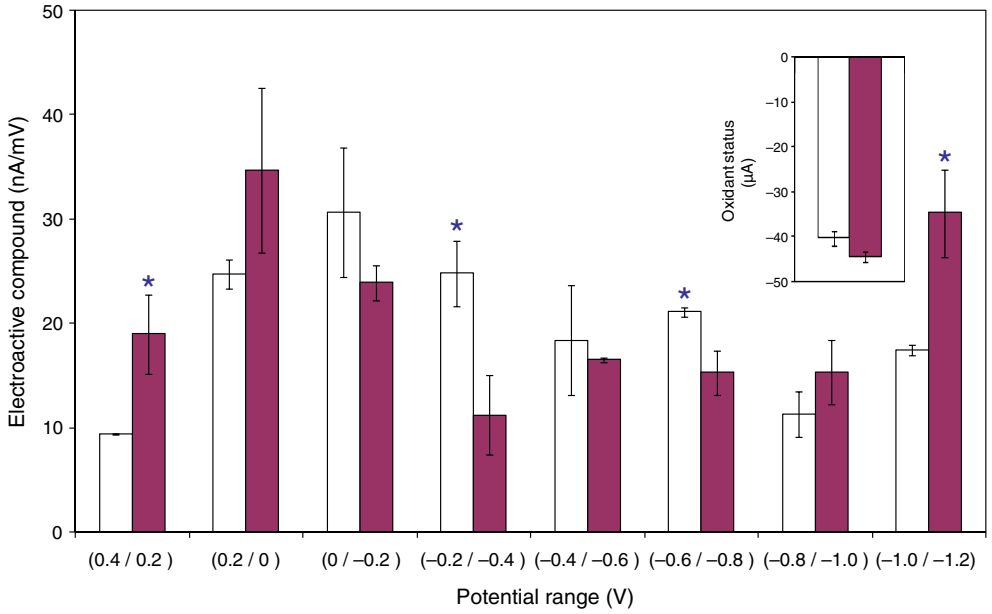

Figure 6 Number of electroactive compounds (nA/mV) in suspensions formed by ovine vaginal cells and *Lactobacillus acidophilus* ATCC 314 supplemented with hydroquinone (□) and metronidazol plus hydroquinone (■), determined from DPV scans within +0.4 and −1.2 V potential ranges. Values were grouped at 0.2 V intervals. The inserted figure shows the *oxidant status* in each of the systems considered. Bars on the columns show the standard error of the mean. An asterisc over the columns indicates that there are significant differences at $p < 0.05$. For further details see Materials and Methods.

treatments were observed at some ranges ($p < 0.05$). Using the concept of oxidant status led to overcome the evident difficulty of interpreting the influence that those partial differences had over adherence, as it helped compare the redox situation systemically. The oxidant status, calculated using the ranges that differed significantly ($p < 0.05$), indicates that this variable had a higher value in the systems supplemented with the H_2Q and MTZ combination (Fig. 6, inserted figure). Moreover, as the expression that defines oxidant status (see Materials and Methods) confirms that if there is a given concentration in an electroactive compound A with an E_1 potential, this same concentration in a compound B with an E_2 potential (being $E_2 < E_1$) will originate a significantly greater oxidant status. Therefore, it may be concluded that although the H_2Q and MTZ combination changes the number of redox compounds compared with H_2Q alone at various potentials, an increase in the redox status will occur due to a concentration increase in the lowest negative potentials within the studied range, that is, between −1.0 and −1.2 V. Based on this analysis, an increase in low-potential compounds should be associated to a decrease observed in the adherence phenomenon. In other words, adherence is reduced when there is an increase in low-potential redox couples which, under normal circumstances in a vaginal system (potentials greater than −1.0 V), should behave as reducers. This result coincides with the results indicated in the bibliography, which show that cell–cell adhesion is induced by oxidant agents[20].

Since MTZ is an antibiotic having redox behavior commonly used in both veterinary and human medicine, this paper aims to demonstrate the importance of considering how the environment in which it must work can be modified and consequently affect the speciation of the molecule, finally influencing bacterial adhesion. Although the redox nature of this complex relationship is not explained in this work, the results show that the study of behavior and fluctuations of the redox compounds that compose the vaginal fluid, in relation to concentration and species[20], must be hierarchized in order to better contribute to understanding the early stages of microbial colonization in animal body cavities, such as the vaginal environment. Because of the results we wonder: if a given strain considered potentially probiotic fails to form biofilm, should not we investigate the redox conditions present in the medium before discarding their effectiveness? And on the other hand: could the redox status be modified through the incorporation of effector molecules in order to prevent pathogenic bacterial colonization?

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interests

The authors declare that there is no conflict of interests

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