

Review Article

Electron Microscopic Studies in *Escherichia Coli* on Mode of Action of Sodium Benzoate and Potassium Sorbate

Norma Angélica Santiesteban-López^{1, *}, Teresa Gladys Cerón-Carrillo¹, José Luis Carmona-Silva¹, Javier Castro-Rosas²

¹Faculty of Administration (Gastronomy). Benemérita Universidad Autónoma de Puebla, University City, Puebla, México

²Academic Area of Chemistry, Institute of Basic Sciences and Engineering, Autonomous University of the State of Hidalgo, Hidalgo, México

Email address:

asantiesteban2@hotmail.com (N. A. Santiesteban-López), teresa.ceronco@gmail.com (T. G. Cerón-Carrillo),

jcarmonas@yahoo.com.mx (J. L. Carmona-Silva), jcastro@uaeh.edu.mx (J. Castro-Rosas)

*Corresponding author

To cite this article:

Norma Angélica Santiesteban-López, Teresa Gladys Cerón-Carrillo, José Luis Carmona-Silva, Javier Castro-Rosas. Electron Microscopic Studies in *Escherichia Coli* on Mode of Action of Sodium Benzoate and Potassium Sorbate. *International Journal of Food Science and Biotechnology*. Vol. 4, No. 4, 2019, pp. 82-86. doi: 10.11648/j.ijfsb.20190404.11

Received: March 11, 2019; Accepted: May 15, 2019; Published: October 24, 2019

Abstract: Traditionally food antimicrobials was utilized to extent the lag phase or inhibit the growth of microorganisms; however, it has been demonstrated that exposure to antimicrobials such as sodium benzoate and potassium sorbate in sublethal concentrations, and gradually increasing the dose, allowed the adaptation of microorganisms of interest in food, such as *E. coli*, exhibiting induced resistance by unknown mechanisms. Therefore, the objective of this study was to identify the ultrastructural changes in viable cells of *E. coli* adapted to high concentrations (7000 ppm) of these antimicrobials, using transmission electron microscopy (TEM). After treatment with potassium sorbate, *E. coli* presented important morphological changes such as the separation of the cell membrane from the cytoplasm and cell wall, the appearance of a remarkable electronic light at the center of cells containing condensed deoxyribonucleic acid (DNA) molecules, as well as the appearance of small dense granules of electrons. Therefore, potassium sorbate induced more severe shape structural changes, presence of unusual structures and loss of integrity compared to viable cells adapted to sodium benzoate.

Keywords: Adaptation, Potassium Sorbate, Sodium Benzoate, *E. coli*, Morphological Changes

1. Introduction

Food antimicrobials are utilized to prolong the lag phase or to inhibit microbial growth, thus, extending food shelf life [1-2]. Antimicrobials can be classified as “natural or traditional”. Natural antimicrobials can be found in microorganisms, plants or animals. Only a few antimicrobial agents come from a natural origin, such as nisine, natamycin, lactoferin, and lysozyme have the approval of international regulatory organisms for their use in food products [1, 3]. On the other hand, among the traditional antimicrobials (chemically synthesized) we can find benzoic and sorbic acids, which use in food products is approved by most of the international regulatory organisms [1]. Sodium and potassium salts respectively,

are used in concentrations below 0.3% to inhibit the growth of several microorganisms. Nevertheless, it has been shown that exposure to antimicrobials such as sodium benzoate and potassium sorbate at gradual increments of concentrations allow for *E. coli* to develop resistance to higher concentrations than those approved by regulatory organisms [3, 4].

Some microorganisms can have a degree of resistance to some stress factors, this resistance can be innate or acquired via mutation or gene exchange, allowing it to grow and survive [5, 2]. There are some scientific studies stating that *E. coli* has several resistance systems to organic acids [6-7] causing genetic, biochemical and physiological changes that have not been fully disclosed as of today. Something similar occurs to many other stress factors to which several

microorganisms have been subjected [8, 2]. To fully understand the resistance to antimicrobial agents, it is necessary to comprehend the action mechanisms and/or the specific sites of interaction between an antimicrobial agent and a specific microorganism. A well-known field, is that of the therapeutic use of antimicrobial agents, which have specific sites of action over the microbial cell and the development of resistance to these agents is the result of alterations in that given specific site and/or the expression of molecules that degrade the antimicrobial [9, 2]. Some therapeutic antimicrobials have as action mechanism the inhibition of the cell wall synthesis, plasmic membrane damage, nucleic acid inhibition, antimetabolites, betalactamases and antifolic inhibitors, among others [1-2].

To understand the resistance mechanisms of *E. coli* in the food industry, it is necessary to research ultrastructural morphological alterations which can be visualized by means of Transmission Electron Microscopy (TEM). Nowadays, there is no reference in the literature regarding the *E. coli* structural cell damage caused by potassium sorbate and sodium benzoate. The aim of the following study is to identify ultrastructural changes in viable.

E. coli ATCC 35218 cells adapted to high concentrations of potassium sorbate and sodium benzoate (7000 ppm) and compare them with non-adapted viable cells.

2. Methods and Materials

2.1. Microorganism

E. coli ATCC 35218 were obtained from Laboratorio de Microbiología de Alimentos de la Universidad de las Américas Puebla. *E. coli* cells were cultivated in Trypticasein Soy Broth (TSB, Difco, Becton Dickinson, Sparks, MD) at $35 \pm 2^\circ\text{C}$, and were kept in Agar Trypticasein Soy slants (TSA, Difco) at $4 \pm 1^\circ\text{C}$.

2.2. Adaptation to Antimicrobial Agents

E. coli ATCC 35218 cells were adapted to concentrations of 7000 ppm of potassium sorbate or sodium benzoate by exposure of the microorganism to increasing incremental concentrations, as described by Santiesteban-López *et al* [10].

2.3. Morphological Analysis of *E. coli* Cells

Adapted cells and non-adapted cells were subjected to three centrifuge cycles and washed with Millonig buffer [11], fixed in a watery solution of osmium tetroxide at 2% (Sigma-Aldrich, St. Louis, USA), for two hours. Cell packages were subjected to a dehydration process in ethanol-acetone with increased concentrations (10 to 80% of ethanol-acetone). Dehydrated packages were included in Spurr resins as done by Someya *et al.* [12], and slices were obtained with Sorvall MT2 ultramicrotome (Dupont Instrumentrs, Newton, USA). The slices were deposited in copper grids, contrasted with Reynolds solution and observed in a Transmission Electron Microscopy Jeol model JEM-1200

EX II (Tokyo, Japan).

3. Results and Discussion

Ultrastructure analysis of non-adapted *E. coli* cells shown the internal structure of basilar bacteria *E. coli*, cells with uniform electronic density were obtained, which suggest that cells are within normal conditions without morphologic alterations. "Figure 1".

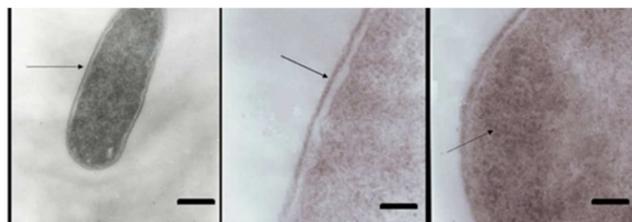


Figure 1. *E. coli* micrography without any morphologic alterations.

Wall and cell membrane are clearly visible, "Figure 1"; cytoplasm shows a normal look where electrodense zones can be observed "Figure 1".

On the other hand, adapted *E. coli* cells generated mechanisms of resistance to antimicrobial agents which are related to morphological changes, in such a way that the observed ultrastructure was different from the non-adapted cells "Figure 2".

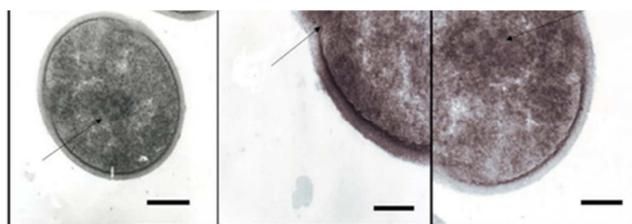


Figure 2. *E. coli* cells treated with 7,000 ppm of Sodium Benzoate.

Microorganisms reproduce in normal physiological environments, any extreme deviation from this causes microbial stress. Prolonged exposure to this stress factors, allows the microorganism to reduce its growth, increases its adaptation time (lag) or totally inactivates [13].

We can appreciate a change in bacterial morphology, the typical basilar shape is modified, the cell acquired a circular shape (arrow). Cell wall is shown to have a continuity alteration "Figure 2". Also, we observed electrodense granules "Figure 2", due to the electron light region, or even, due to the collapse of the cell wall. Many gram positive and negative bacterias are capable of withstanding this change, being able to adapt and become resilient [14, 3, 7]. Previous studies demonstrated that *E. coli* adaptation to sodium benzoate or potassium sorbate at concentrations of 7000 ppm [10].

Comparing our results for *E. coli*, with those previously obtained in *P. aeruginosa*, Koike *et al.* [15-16] shows a correlation between the antimicrobial agent resistance (polymyxin 25 μg) and a cellular structure alteration of *P. aeruginosa*. Our findings reinforce the possibility that

morphological changes exhibited by *E. coli* are caused by adaptation mechanisms to high concentrations of sodium benzoate to which they were exposed.

In a similar study, Whan et al. [17-18, 7] observed that exposition of *E. coli* to elevated concentrations of polymyxin (> 200µg), produced protuberances in cell surface and the appearance of extended foldings in the cytoplasmic membrane. Barret and Asscher [8] suggest that the development of resistance to carbenicillin of *P. aeruginosa* is caused by a modification of type and or quantity of lipopolysaccharids (LPS) of the outer wall. Other studies confirm the resistance of *P. aeruginosa* is caused by the differences of LPS compositions and a complementary mechanism of small porins that stops the traffic of some substances by diffusion. Presence of less acid LPS in the outer wall can be a factor that contributes to the intrinsic resistance [19, 3]. Other studies have proposed that *E. coli* ampicillin resistance could be caused by alterations of LPS composition [20, 2].

Similarly, Koike *et al.* [15, 7] concluded that antimicrobials (such as ampicillin) produce an effect upon Gram negative cells like *E. coli*, due to the disorganization of LPS, which in turn, causes a formation of outer wall projections. Those studies reinforce what we found in the present research; nevertheless, there is still need of an analysis of structural composition of wall and cellular membrane, to give conclusive evidence about the relation of morphological and structural changes induced by the adaptation of *E. coli*.

On the other hand, the growth of *E. coli* cells adapted to 7000 ppm of potassium sorbate cause severe morphological changes, even more noticeable than those observed with sodium benzoate "Figure 3".

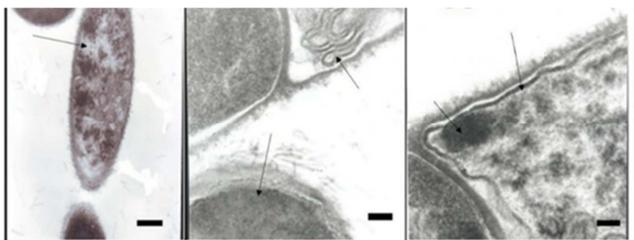


Figure 3. *E. coli* cells treated with 7.000 ppm of Potassium Sorbate.

Bacterial cells adapted to sublethal concentrations of potassium sorbate is shown in figure 3, a notable region of electronic light appeared (arrow) in those cells treated with potassium sorbate, this phenomenon tends to appear in cells treated with some antimicrobial substances such as silver, for example *E. coli* and *S. aureus* [6, 3, 21]. As it is known, the light region and the wall cell can collapse and electrodense granules can get into the cell. In figure 3 we show important morphological changes, cell membrane is contracted and separated from the cell, cell wall has been severely damaged (long arrow). It can be seen a condensed DNA shape within the centre region of electronic light (short arrow). In figure 3 we can observe a long space between cytoplasm, wall and cell membrane (long arrow), also, we can observe

electrodense granules (short arrow). Similar morphological changes were reported by Someya et al. [12, 2], by exposing *E. coli* with high concentrations (0.5 ppm) of bicyclomycin, achieving an inhibition of phragmoplast formation and filamentous cells generation, some with morphological alterations such as cytoplasmic material agglutination and the presence of dark bodies [6].

Tanaka et al. [22-23] found that bicyclomycin inhibit the biosynthesis of cell wall lipoproteins in the coupling site within the internal membrane of *E. coli*. Uri and Actor [24-25], shown by means of optical microscopy that enoxacin (0.5 ppm) produces morphological alterations in *E. coli* similar to those obtained in our study with potassium sorbate, but for lower concentrations. Both antimicrobials caused an elongation of cell body.

Changes observed in our study were compared with those obtained by Feng et al. [6], which reported that *E. coli* and *S. aureus* treated with AgNO₃ and analysed with transmission electronic microscopy shown morphological changes in *E. coli*, such as cell membrane, cell wall and cytoplasm separation, and DNA concentration.

Previous ultrastructural studies done by Koike *et al.* [15, 7], demonstrated the effect of polymyxin in resistant *P. aeruginosa* strains, in which analysed cells shown projections from the outer layers and external cell membrane as small vesicles, membranous structures accumulation and cytoplasmic electrodense zones, comparable changes to those shown by *E. coli* adapted cells to sodium benzoate and potassium sorbate [26].

By relating the morphological changes observed in *E. coli* in the present study, adapted *E. coli* cells to sodium benzoate and potassium sorbate, with the growth curves reported by Santiesteban-López et al. [10], in which it is shown that *E. coli* cells exposed to 7000 ppm of sodium benzoate exhibited a short adaptation phase (lag), less than 1 h, this shows a faster adaptation to the stress factor, which in turn could be proven with minutes changes regarding shape and cellular integrity. In a similar fashion, cells adapted to potassium sorbate exhibited drastic changes in their ultrastructure, maybe caused by a metabolic pathway modulation or mechanisms generated to adapt to the antimicrobial agent (potassium sorbate), which causes a longer adaptation phase of 2 h, i. e. a longer lag time than those cells adapted to sodium benzoate [27-28].

4. Conclusions

Ultrastructural study of *E. coli* cells adapted to high concentrations of sodium benzoate or potassium sorbate shown evidence of changes and alterations in the microorganism morphology.

By comparing the ultrastructural changes of *E. coli* cells adapted to 7000 ppm of potassium sorbate and sodium benzoate, we observed that potassium sorbate induces more drastic changes than those caused by sodium benzoate adapted cells. Generally speaking, these were shape changes and cellular integrity loss. All these phenomena suggest that

sodium benzoate has a minor effect over the *E. coli* cell structure than potassium sorbate.

The only structures observed in the *E. coli* cells adapted to sodium benzoate were abundant electrodense granules. However, with potassium sorbate we also observed the presence of electrodense granules, a cell membrane separated from the cell wall and the cytoplasm, and severe damages in the cell wall, but not reaching cellular lysis.

These findings suggest that the exposure of this microorganism to elevated concentrations of this antimicrobial agents has an effect on the ultrastructure of this bacteria. Direct observations, however, could not provide enough information about how these antimicrobials exert its actions over *E. coli* and how the resistance to these agents is acquired without causing cell death. It is necessary to perform studies in which the cell wall composition is determined, besides a further study about the ultrastructure, so it can be identified the active site or the mechanism by which, each antimicrobial exerts its action in a specific manner.

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