Biotecnología

Revista Mexicana de Ingeniería Química

Vol. 14, No. 2 (2015) 347-354

CHANGES IN MICROSTRUCTURE OF *Salmonella* TYPHIMURIUM AND *Listeria monocytogenes* EXPOSED TO HYDROXYCINNAMIC SALTS

CAMBIOS EN LA MICROESTRUCTURA DE *Salmonella* TYPHIMURIUM Y *Listeria monocytogenes* EXPUESTAS A SALES HIDROXICINÁMICAS

D. Martínez-Arámburu, G.K. González-Quijano, L. Dorantes-Alvarez*, G. Aparicio-Ozores, E.O. López-Villegas

Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. Prolongación de Carpio y Plan de Ayala. Col. Sto. Tomás, México, Distrito Federal, C.P. 11340. México.

Received November 15, 2014; Accepted March 19, 2015

Abstract

Bacteria are microorganisms that contain organelles, which have sizes in the nanoscale range. Certain stress conditions may induce morphological changes in bacteria as a strategy to survive. In the present work, the effects of two antimicrobials commonly found in plants were tested to determine the ultrastructure and morphology of *Salmonella* Typhimurium and *Listeria monocytogenes*. Kinetics of survival of bacteria were monitored, and ultrastructure changes were observed using scanning and transmission electron microscopy (SEM and TEM, respectively). The results show a bacteriostatic effect on *Salmonella* at sodium ferulate concentrations between 0.3 and 0.6%, which also caused elongated forms of the bacteria, which were observed by scanning electronic microscopy (SEM). The Feret diameter and surface area of the bacteria were determined using image analysis, where it was shown that these dimensions increased four times on average compared with those of the control cells. These data support the idea of multicellular aggregates. In the case of *Listeria monocytogenes*, the elongation of the cells occurred after 24 hours of exposure to 0.6% sodium ferulate. The number of cytoplasmic inclusions and the size of vacuoles increased after the first hour of exposure to 0.8% sodium caffeate and corresponded to a bactericidal effect of this antimicrobial. The elongation of the cells that occurred in bacteriostatic concentrations reverted when the bacteria were incubated in media without the antimicrobials, and the kinetics of growth became normal, which suggests a strategy to survive under stress conditions generated by the presence of antibacterial compounds.

Keywords: ultrastructure, *Salmonella* Typhimurium, *Listeria monocytogenes*, morphological changes, phenolic acids.

Resumen

Las bacterias son microestructuras biológicas que contienen organelos en rangos nanométricos. Algunas condiciones de estrés pueden inducir cambios morfológicos en las bacterias como una estrategia de supervivencia. En el presente trabajo, el efecto de dos antimicrobianos comúnmente encontrados en plantas fueron probados en la ultraestructura y en la morfología de *Salmonella* Typhimurium y *Listeria monocytogenes*. Las cinéticas de supervivencia de las bacterias fueron monitoreadas y los cambios en la ultraestructura fueron observados usando microscopía electrónica de barrido (SEM) y electrónica de transmisión (TEM). Los resultados mostraron un efecto bacteriostático sobre *Salmonella* a concentraciones de 0.3 y 0.6% de ferulato de sodio que causaron cambios morfológicos en las bacterias y que fueron observados por SEM. El diámetro de Feret y el área de las células fueron determinados empleando análisis de imágenes. En el caso de *Listeria monocytogenes* la elongación celular ocurre después de 24 horas de exposición a 0.6% de ferulato de sodio. Las inclusiones citoplasmáticas y vacuolas incrementaron después de 1 hora de incubación con 0.8% de cafeato de sodio. La elongación de las células causada en condiciones bacterióstaticas fue revertida cuando las bacterias fueron incubadas en medio sin los antimicrobianos y las cinéticas de crecimiento fueron normales, implicando un mecanismo de supervivencia bajo condiciones de estrés.

Palabras clave: microestructura, *Salmonella* Typhimurium, *Listeria monocytogenes*, cambios morfológicos, ácidos fenólicos.

*Corresponding author. *E-mail*: ldoran@ipn.mx
Tel. 57-29-60-00, X 62467

1 Introduction

Evaluation of ultrastructures of encapsulates and nanostructures using image analysis may help to explain various phenomena. Image analysis methods have been applied to quantitatively evaluate the morphology and texture of microencapsulates and certain microorganisms by extracting information from images captured with different acquisition systems. The extracted information has been useful to characterize changes in the morphological features, such as the minor axis and aspect ratio, of *Lactobacillus casei* Shirota due to the effect of different bile salts (Gonzalez-Vázquez *et al*., 2014). Furthermore, diseases caused by the ingestion of contaminated food are a global health problem regardless of the advances in public health and the conservation of food products, which occur primarily in zones with poor hygienic conditions, e.g., in the production chain of milk products (Morales-Pablo *et al*., 2012). *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* represent a concern with regard to food safety due to its ability to grow in a wide range of adverse environmental conditions. *S*. Typhimurium has been implicated in outbreaks caused by the ingestion of food products, such as chicken and ground meat (CDC, 2014), and has displayed an unusual tolerance to factors used to control bacterial growth in foods, such as acidity and NaCl addition. *L. monocytogenes* is a pathogen of concern in refrigerated food products, such as cheese and milk. It is therefore necessary to control its growth to overcome its ability to survive or even to develop adaptation mechanisms under various severe environments (Bereksi *et al*., 2002; Apostolidis *et al*., 2008). Among the barriers used to prevent bacteria survival is the use of preservative agents that have antimicrobial activity. Several of these agents are natural compounds derived from plants, including spices and essential oils (Hernández-Ochoa *et al*., 2011). Hydroxycinnamic acids are a group of phenolic derivatives that are present in rice, corn, coffee, eggplant, cabbage, spinach and broccoli. Several of these agents are also found in peppers and have shown antibacterial activity against *Staphylococcus aureus*, *Salmonella* Typhimurium, *Listeria monocytogenes*, and *Bacillus cereus* (Dorantes *et al*., 2000). An inhibitory effect has been demonstrated by measuring the halos around paper disks containing chili extracts or phenylpropanoids, which were placed on agar plates with the bacteria. The solubility of these compounds improved when sodium salts were prepared and assayed against *Escherichia coli* (Dorantes-Alvarez *et al*., 2011). However, studies on the adaptation of pathogenic bacteria to natural antimicrobials are required to be able to recommend their use in appropriate concentrations to control pathogenic bacteria in food processing. Therefore, the objective of this work was to investigate the effect of sodium ferulate and sodium caffeate on the survival and ultrastructure of *S*. Typhimurium and *L. monocytogenes*.

2 Materials and methods

2.1 Bacteria and culture conditions

Salmonella enterica serovar Typhimurium (ATCC 14028) and *Listeria monocytogenes* (ATCC 19115) were obtained from the Medical Bacteriology Laboratory of the Escuela Nacional de Ciencias Biológicas (IPN, Mexico City, Mexico) and were maintained on trypticase soy agar plates (TSA, BD Bioxon, México City, Mexico) at 4°C until use. The strains were sub-cultured monthly to insure their viability.

2.2 Kinetics of survival of S. Typhimurium and L. monocytogenes

Ferulate dilutions at concentrations of 0.15, 0.3, 0.6, 1.2, 2.5 and 5% were prepared to evaluate their inhibitory activity on *S*. Typhimurium and *L. monocytogenes*. The dilutions were adjusted to a pH of 7, sterilized and inoculated with a suspension of the bacteria to obtain 10^4 colony-forming units (CFU/mL). The kinetics were determined after 12, 24, 36 and 96 hours. Controls without antimicrobials were prepared for each assay. The number of colony-forming units were then counted. All of the experiments were performed in triplicate, and the optimum growth/death rate was calculated. In the case of the kinetics of the survival/death of both bacteria in the presence of caffeate, the same concentrations were assayed; however, the time of incubation were shorter than those used for the effect of ferulate because at the same concentrations, caffeate kills bacteria within a few hours.

2.3 Analysis of morphometric changes of S. Typhimurium and L. monocytogenes subjected to treatment with ferulate and caffeate by optical microscopy

Samples of *S*. Typhimurium and *L. monocytogenes* with and without antimicrobial treatment were stained with fuchsin dye for one minute and were observed using an optical microscope (Carl Zeiss, Axio Scope A1, Oberkochen, Germany).

2.4 Analysis of the morphometric changes of S. Typhimurium and L. monocytogenes subjected to treatment with ferulate and caffeate by scanning electron microscopy (SEM)

Cultures of *L. monocytogenes* and *S*. Typhimurium were adjusted to the 0.5 McFarland nephelometer tube, where 0.1 mL of inoculum was placed in 5 mL of TSB with sodium caffeate at concentrations of 0.3 and 0.15 and incubated at 37°C for 2 and 24 hours, respectively. For the sodium ferulate, concentrations of 0.3 and 0.6% were chosen. SEM analysis was performed using an electronic microscope (Science-EMS, JEOL model JSM-5800LV, Washington, D.C., USA) and following the conventional methodology for sample preparation. The samples were fixed with gluteraldehyde 2% for one hour. Then, the samples were washed 3 times with a buffer. Each sample with osmium tetroxide was set at 1% in the same buffer for 1 hour. Then, the samples were washed with increasing ethanol-water solutions of 40-90% for 10 min and then finally washed with 100% ethanol. The samples were dried by the principle of critical point and set in conductive tapes. Finally, the samples were coated with gold and examined under the scanning electron microscope.

2.5 Image analysis

After acquiring the images, which were randomly chosen, micrographs from each ferulate or caffeate treatment and the control samples were analyzed using the software ImageJ, v. 1.49p, (National Institutes of Health, Bethesda, Maryland, USA), to obtain the morphometric parameters of the bacteria. The Feret diameters of the treated samples and control samples and the area of the bacteria were calculated.

2.6 Statistical analysis

All data of the control and treated bacteria were analyzed by one-way analysis of variance using the software Minitab 15.1.30.0. Inc. at a 0.05 significance level. Significance differences among the samples and controls were determined using the Dunnet test.

2.7 Analysis of the ultrastructure of Listeria and Salmonella subjected to treatment with ferulate and caffeate by transmission electron microscopy (TEM)

L. monocytogenes was adjusted in the 0.5 McFarland nephelometer tube, which corresponded to 10^4 CFU/mL. The inoculum was placed in 20 mL of TSB and allowed to grow at 37°C for 24 hours to ensure there were enough bacteria for TEM analysis. Afterwards, sodium caffeate was added to give final concentrations of 0.6, 0.8 and 1.2%, which were incubated at 37°C for 2, 4 and 6 hours, respectively. The same procedure was performed with the *Salmonella* culture. TEM analysis was performed following the conventional methodology for sample preparation. The samples were fixed with gluteraldehyde in a 2.5% phosphate regulator for 1 hour and washed 3 times with a buffer. Each sample with osmium tetroxide set at 1% in the same buffer for 1 hour. Then, the samples were washed with increasing ethanol-water solutions of 40-90% for 10 min, and finally, the samples were washed with 100% ethanol. Samples of Epon resin mixtures with propylene-oxide were created at ratios of 1:2, 1:1 and 3:1 for 2 hour each. Finally, the resin included 100% Epon with two 2-h changes each. The samples were identified and allowed to incubate at 60°C for 24 hours to polymerize the resin. The polymerized samples were cut with an ultramicrotome Leica Ultracut (UCT), contrasted with uranyl acetate and lead citrate and examined under the transmission electron microscope JEOL (JEM-1010, 60 kV, Electron microscopy Science-EMS, Washington, D.C., USA)

3 Results and discussion

3.1 Kinetics of growth/survival/death of the bacteria

Figures 1 and 2 show the kinetics throughout the seventy-two hours of incubation of *S.* Typhimurium and *L. monocytogenes* in the presence of seven concentrations of ferulate are shown, respectively. Additionally, the growth of the bacteria in the controls without ferulate is also shown. The biological parameters λ (lag phase), μ (rate of growth or death), and A (maximum growth) were calculated and are shown in Table 1 and Table 2. The growth rate of *S.* Typhimurium in the control (without ferulate) was 0.25 h^{-1}, with a maximum growth of 10^8 CFU after 72 hours of incubation. When *Salmonella* was incubated in the media with 0.3% and 0.6% of ferulate, the growth rate decreased significantly to 0.051 and 0.011 h^{-1}, and the population increased only by 1.3 and 0.6 logs CFU, respectively, when compared with the population at the beginning of the experiment (Fig. 1). Therefore, 0.3% and 0.6% of ferulate were considered to cause a bacteriostatic effect against *S.* Typhimurium. When the ferulate concentration was of 0.8%, a rapid decrease in the population was observed, and the death rate was -0.60 h^{-1}. Similar rates of bacteria death (-0.6, -0.59, 0.63 h^{-1}) were observed with 1.2, 2.5 and 5% of ferulate. This was considered a bactericidal effect against *S.* Typhimurium. Statistical analysis by ANOVA and the Dunnet test corroborate the significant differences (p <0.05) between the control and samples with 0.3% or higher ferulate concentrations

When *Listeria monocytogenes* experienced different concentrations of ferulate, the bacteriostatic effect was evident at 0.3 and 0.6% of ferulate.

Table 1. Biological parameters of *S.* Typhimurium in the presence of ferulate

Ferulate concentration (%)	λ (h)	μ (h^{-1})	A (log CFU) 24 hours
0	6	0.25	8.8
0.15	6	0.19	8.5
0.3	12	0.051	5.5
0.6	24	0.011	4.67
0.8	6	-0.60	0
1.2	0	-0.60	0
2.5	0	-0.59	0
5	0	-0.63	0

Additionally, a bacteriostatic effect was observed with concentrations of 0.8 and 1.2% of *Listeria* until 48 hours of incubation; afterwards, a decrease in the *Listeria* population occurred, and at 72 hours, the bacteria count was zero. Therefore, the lag phases of *Listeria* in both concentrations were 48 hours (see Table 2). When a 2.5% concentration was used, the bactericidal effect was registered after 24 hours and with a 5% concentration, after twelve hours. These lag phase values are also shown in Table 2. In both bacteria, it may be concluded that growth rate decreases as the concentration of sodium ferulate increases, and thus, a dose-response effect was observed, where *L. monocytogenes* was more resistant than *S.* Typhimurium at 0.8 and 1.2% of ferulate. Statistical analysis by ANOVA and the Dunnet test corroborate the significant differences (p <0.05) between the control and samples with 0.3% or higher ferulate concentrations.

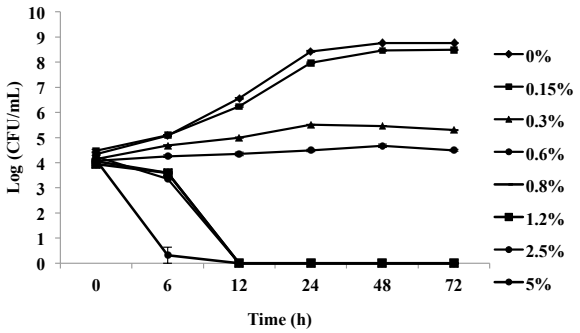

Fig. 1. Survival/death/kinetics of *S.* Typhimurium treated with different concentrations of sodium ferulate.

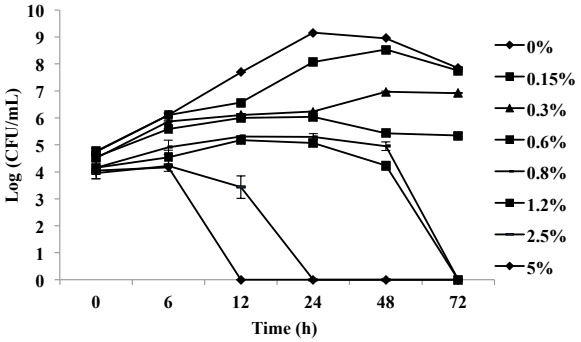

Fig. 2. Survival/death kinetics of *L. monocytogenes* treated with different concentrations of sodium ferulate.

Table 2. Biological parameters of *L. monocytogenes* in the presence of ferulate

Ferulate concentration (%)	λ (h)	μ (h^{-1})	A (log CFU) 24 hours
0	0	0.26	9.15
0.15	0	0.126	8.5
0.3	0	0.031	6.9
0.6	0	0.021	6
0.8	48	0.020	5.4
1.2	24	0.017	5.1
2.5	6	-0.300	0
5	6	-0.693	0

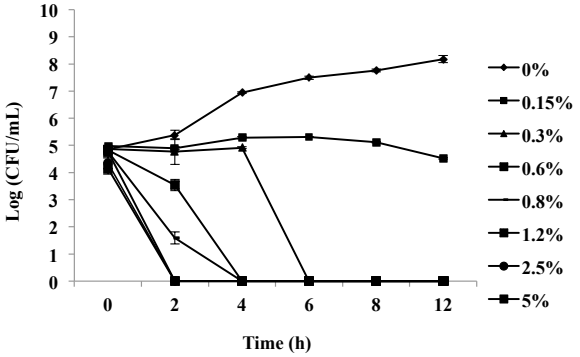

Fig. 3. Survival/death kinetics of *S.* Typhimurium treated with different concentrations of sodium caffeate.

Table 3. Biological parameters of *S.* Typhimurium in the presence of caffeate

Caffeate concentration (%)	λ (h)	μ (h^{-1})	A (log CFU) 12 hours
0	2	0.29	8.17
0.15	2	0.19	5.21
0.3	4	-2.45	0
0.6	0	-1.80	0
0.8	0	-1.65	0
1.2	0	-2.35	0
2.5	0	-2.17	0
5	0	-2.07	0

When the bacteria were challenged with the seven different concentrations of sodium caffeate, the time of the experiments were reduced compared with those assayed with ferulate. The reason is that caffeate is more efficient in killing *Salmonella* and *Listeria*; thus, the longest time of incubation was twelve hours.

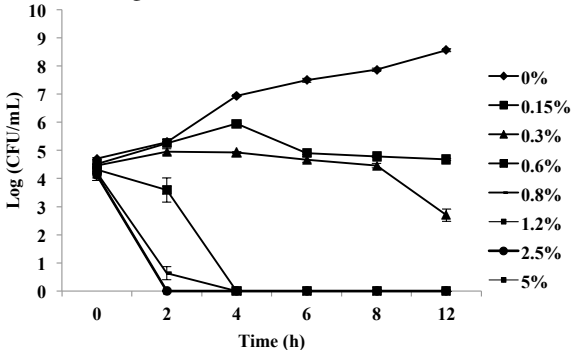

Fig. 4. Survival/death kinetics of *L. monocytogenes* treated with different concentrations of sodium caffeate.

Table 4. Biological parameters of *L. monocytogenes* in the presence of caffeate

Caffeate concentration (%)	λ (h)	μ (h^{-1})	A (log CFU) 12 hours
0	2	0.33	8.55
0.15	2	0.05	5.1
0.30	8	-0.50	2.9
0.60	0	-0.60	0
0.80	0	-1.60	0
1.20	0	-2.07	0
2.50	0	-2.06	0
5	0	-2.06	0

The kinetics of the survival/death of *Salmonella* are shown in Figure 3, where a bacteriostatic effect of caffeate was observed only at 0.15%, whereas bactericidal effects were observed at 0.3, 0.6, 0.8, 1.2, 2.5 and 5.0% after a lag phase of 4, 2, 2, 0, 0, and 0 hours, respectively (Table 3). Statistical analysis by ANOVA and the Dunnet test corroborated the significant differences (p <0.05) between the control and samples with 0.15% or higher caffeate concentrations.

The survival/death kinetics of *Listeria* in the presence of caffeate (Fig. 4) is extremely similar to *Salmonella*, with a difference at 0.3%, where the lag phase was eight hours before the decrease in bacteria concentration to 1.5 log CFU after twelve hours (Table 4). Statistical analysis by ANOVA and the Dunnet test corroborate the significant differences (p <0.05) between the control and samples with 0.15% or higher caffeate concentrations.

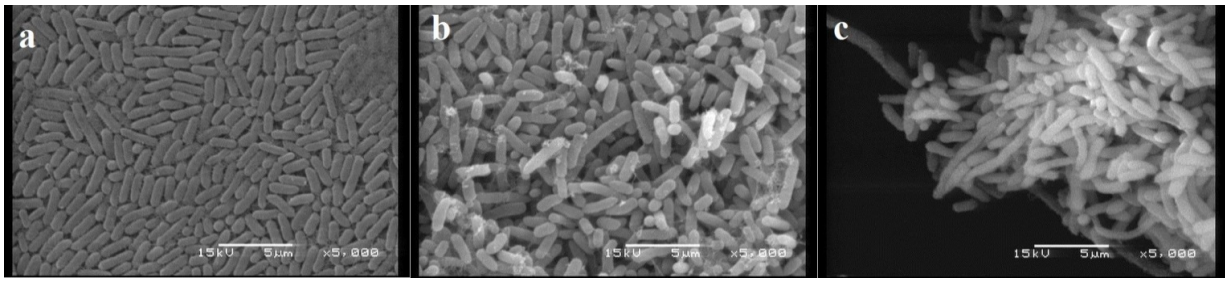

Fig. 5. SEM micrograph of *S.*Typhimurium. 5a: Control cells after 24 h of incubation; 5b: Cell elongation phenomenon growing with the presence of 0.3% of ferulate in triptych soy broth after 24 h; 5c: Cell elongation phenomenon growing with the presence of 0.3% of ferulate in triptych soy broth after 24 h. Bar indicates 5 μm.

Table 5. Average area (μm^2)* and Feret diameter* (μm) of the bacteria cells after incubation with 0.3 and 0.6% of sodium ferulate for *S.* Typhimurium and *L. monocytogenes*

Microorganism	Area (μm^2)			Feret diameter (µm)		
	Control	0.3% treatment	0.6% treatment	Control	0.3% treatment	0.6% treatment
S. Typhimurium after 24 hours	0.98 ± 0.03	4.60 ± 0.92 (6%)	4.16 ± 0.94 (39%)	1.99 ± 0.05	6.62 ± 1.16 (6%)	6.62 ± 1.22 (39%)
L. monocytogenes after 24 hours	0.60 ± 0.02	0.66 ± 0.04 (1%)	2.21 ± 0.41 (12%)	1.58 ± 0.01	1.57 ± 0.10 (1%)	3.65 ± 0.43 (12%)

* Average ± standard error. Values in parenthesis refer to the percentage of atypical cells.

3.2 Morphometric parameters of Salmonella and Listeria affected by sodium ferulate

Optical and SEM micrographs of S. Typhimurium and *L. monocytogenes* were obtained under bacteriostatic concentrations of ferulate to observe changes in the morphometric parameters of the bacteria. Figure 5 shows the micrographs obtained by scanning electronic microscopy for *Salmonella*. The morphology of the bacteria cells incubated in the control media without ferulate agrees with the typical size and shape of bacilli. However, elongated forms were observed when S. Typhimurium and *L. monocytogenes* were incubated with sodium ferulate at 0.3% and 0.6% (Figure 5).

To evaluate quantitatively the number of atypical bacteria and measure the Feret diameter and the area of the bacteria, six SEM micrographs of each control and treated samples were analyzed by Image Analysis. Significant differences (p <0.05) were observed in the size of *S.* Typhimurium cells without ferulate compared with those of the atypical (elongated) cells treated with different concentrations of ferulate for 24 hours. The Feret diameter ratio of treated bacteria with respect to the control was calculated to determine how much larger the elongated cells were with respect to the control cells; the values for the control cells was 1.99 ± 0.05 μm, and the average for the cells treated with 0.3% ferulate was 6.62 ± 1.16 μm and for 0.6 % ferulate, was 6.62 ± 1.22 μm. The area of the *Salmonella* bacteria of the control samples increased by approximately four times in the ferulate-treated samples (Table 5). Morphological modifications have been reported by Philips *et al*., (1998); these authors observed that chilling induced cell elongation that corresponded to 90 % sublethal injury in *Salmonella enteritidis*. The authors suggested a possible link between cell elongation (up to 15 μm) and heat and acid-tolerance as a pathogenicity strategy.

In the case of the Feret diameter of *Listeria* cells, the average value was 1.58 in the control, which means the bacteria are smaller than *Salmonella* cells. There was no significant difference when *Listeria* was treated with 0.3% of ferulate compared with the control; this may be because *Listeria* was more resistant to ferulate than *Salmonella*. This may be observed in the kinetics when the number of bacteria increased after 24 hours with treatment with ferulate 0.3%. When treated with 0.6% ferulate, a significant increment of 3.65 was observed. The area of the atypical cells also increased from 0.60 to 2.21. The increase in the Feret diameter and the surface area of the bacteria in the stress media supports the idea of the formation of multicellular

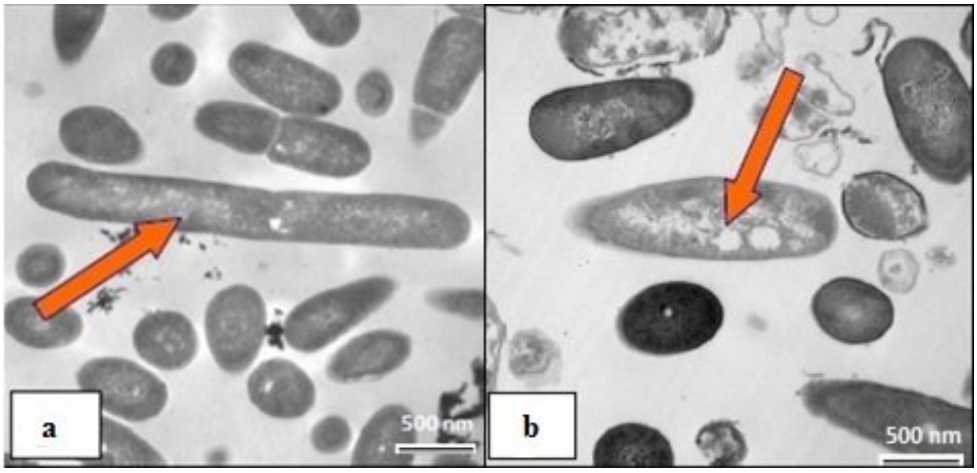

Fig. 6. TEM micrograph of *L. monocytogenes*. 6a: Cell elongation phenomenon growing with the presence of 0.8% of caffeate in triptych soy broth after 30 min; 6b: Cytoplasmic inclusions and size of vacuoles increased after 60 min of incubation with caffeate at 0.8%. Bar indicates 500 nm.

aggregates in response to the presence of ferulate. In other reports, it was found that *L. monocytogenes* cells exposed to acid or alkaline media underwent morphological changes that increased as the growth environment became more challenging (Phan *et al*., 2000; Giotis *et al*., 2007). These changes may be due to a stressful environment and an adaptation of these species as a survival strategy. In broader terms, the development of stress-induced variations in microbial morphology may reflect a wider range of phenotypic adaptations in *L. monocytogenes* (Giotis *et al*., 2007). In the present work, the removal of such stresses (bacteriostatic concentrations) resulted in the return to normal cell forms and growth rates (0.24-0.26 h^{-1}); thus, it is important in food preservation to maintain bactericidal concentrations to achieve bactericidal effect and prevent adaptation phenomena.

3.3 Ultrastructure of Listeria monocytogenes in the presence of sodium caffeate

The TEM micrographs showed the phenomenon of elongation after 30 min of incubation with 0.8% sodium caffeate. Additionally, the Feret diameter increased in average by 3 compared with the normal size. These changes occurred during the first 30 min of incubation, which implies a faster inhibitory effect of this salt (Fig. 6a). Additionally, when *L. monocytogenes* was exposed for one hour to sodium caffeate at 0.8%, the cytoplasm degraded, which increased the formation of nanostructures, such as vacuoles and other cytoplasmic inclusions. The loss of the cell membrane integrity was observed in several samples (Fig. 6b). The increases in size and number of vacuoles as a response to caffeate were also observed in *Salmonella*.

Conclusions

These results indicate that sodium ferulate and caffeate have the potential as antibacterial agents when used in appropriate concentrations to inactivate *S*. Typhimurium and *L. monocytogenes* to increase biosafety of consumable food products. The sodium salts of ferulic and caffeic acids produced a bacteriostatic or bactericidal effect depending on the dose. The cells of *S*. Typhimurium and *L. monocytogenes* exposed to hydroxycinnamic salts in sublethal conditions experienced morphological changes, such as elongated microstructures. The Feret diameter and the area of these elongated forms support the idea that these are multicellular aggregates. Additionally, it was observed that nanostructures, such as vacuoles, increased in size and number.

Acknowledgements

The authors thank CONACYT and PIFI-IPN for fellowships that support this Ph.D research in Food Science in the ENCB-IPN. This work was supported by IPN grants SIP 20150829 and 20141394.

References

Apostolidis, E., Kwon, Y.I and Shetty, K. (2008). Inhibition of *Listeria monocytogenes* by oregano, cranberry and sodium lactate combination in broth and cooked ground beef systems and likely mode of action through proline metabolism. *International Journal of Food Microbiology 128*, 317-324.

Bereksi, N., Gavini, F., Benezech, T., and Faille, C. (2002). Growth, morphology and surface properties of *Listeria monocytogenes* Scott A and L028 under saline and acid environments. *Journal of Applied Microbiology 92*, 556-565.

Center for Disease Control and Prevention USA (CDC). (2014). Available at: www.cdc.gov

Dorantes, L., Colmenero, R., Hernández, H., Mota, L., Jaramillo, M.E., Fernández, E., and C. Solano. (2000). Inhibition of growth of some foodborne pathogenic bacteria by *Capsicum annum* extracts. *International Journal of Food Microbiology 57*, 125-128.

Dorantes, L., Aparicio, G., Ramirez, A. (2011). Improving water solubility of natural antibacterials to inhibit important bacteria in meat products. *Procedia Food Science 2011*, 173-177.

Giotis, E.S., Blair, I.S., McDowell, D.A. (2007). Morphological changes in *Listeria monocytogenes* subjected to sublethal alkaline stress. *International Journal of Food Microbiology 120*, 250-258.

Gonzalez-Vázquez, R., Gutierrez-López, G.F., Arellano-Cárdenas, S., López-Villegas, E.O., Téllez-Medina, D.I and Rivera-Espinoza, Y. (2014). Morphometric Parameters, Zeta potential and growth rate of *Lactobacillus casei* Shirota by effect of different bile salts. *Revista Mexicana de Ingeniería Química 13*, 189-199.

Hernández-Ochoa, L., Gonzales-Gonzales, A., Gutiérrez-Méndez, N., Muñoz-Castellanos, L.N and Quintero-Ramos, A. (2011). Study of the antibacterial activity of chitosan-based films prepared with different molecular weights including spices essential oils and functional extracts as antimicrobial agents. *Revista Mexicana de Ingeniería Química 3*, 455-463.

Morales-Pablo, R., Avalos de la Cruz, D.A., Leyva-Ruelas, G. Ybarra-Moncada, Ma.C. (2012). Bacteriological quality goat raw milk produced in Miravalles, Puebla. *Revista Mexicana de Ingeniería Química 11*, 45-54.

Phan Thanh, L., Mohouin, F., and Aligé, S., (2000) Acid responses of *Listeria monocytogenes*. *International Journal of Food Microbiology 55*, 121-126.