EXACERBATION RISK ANALYSIS OF CERTAIN POTENTIAL HUMAN PATHOGENS IN COW MILK ACTIVATED WITH THE LACTOPEROXIDASE SYSTEM

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Abstract
The Lactoperoxidase system (LPS) consists of the lactoperoxidase enzyme, thiocyanate, and hydrogen peroxide and has a broad antimicrobial activity. There are experimental and practical evidences demonstrating that the use of this method is innocuous. However, CODEX guidelines have identified the need of having greater information about the risk of exacerbation of pathogen bacteria present in milk because of the inhibition of the natural flora present in raw milk. The objective of the present study consisted on evaluating the effect of the LPs activation on the exacerbation of certain potential human pathogens: *Salmonella typhimurium.*, *Staphylococcus* ***aureus,*** *Escherichia coli 0157:H* and *Listeria monocytogenes* in cow raw milk activated with the Lactoperoxidase system. The experiment was carried out at the National Center for Animal and Plant Health and the Food Nutrition and Hygiene Institute in Cuba. This study was carried out for each pathogen in both laboratories, in parallel, with the same milk samples and under the same conditions and analytical diagram. Analyses were made using the techniques established for the enumeration of these pathogens. As carriers of the LPs active principles, a product named STABILAK was used, bringing an equivalent quantity of 9 mg/L of sodium thiocyanate and 34 mg/L of sodium percarbonate. Test times per each replica in milk treated with the product and control milk were 0, 4, 8 and 12 hours respectively. The LPs effects observed at 12 hours, according to the *Staphylococcus* ***aureus*** and ***Salmonella typhimurium*** determination***,*** did not show significant differences in the counting in activated milk with respect to the control. *Listeria monocytogenes* and *Escherichia coli O 157:H7* showed a significant reduction ($P<0.05$). In all the cases, the control sample showed a higher growth than the activated milk. The general behavior indicates that there was no exacerbation of the pathogens studied. That is why; there is not any microbiological risk for the consumer, associated to the use of this preservation method.
Key words: LP system; foodborne pathogens; risk analysis

Introduction
The Lactoperoxidase system (LPS) consists of the lactoperoxidase enzyme (LP), thiocyanate (SCN-), and hydrogen peroxide (H_20_2) and has a broad antimicrobial activity (23, 33, 39). The LP-catalyzed oxidation of SCN- by H_20_2 generates hypothiocyanite anion ($OSCN^-$) which is in equilibrium with hypothiocyanous acid, PKa 5.3 (1,40). The hypothiocyanous acid plus OSCN- can oxidize essential sulfhydryl groups (-SH) in metabolic enzymes, thereby inhibiting bacterial growth (6,42). Structural damage or changes in bacterial membranes with subsequent leakage or impaired uptake of nutrients, or both, have also been reported (7, 39).

The Lactoperoxidase System for raw milk preservation is currently the only approved method, apart from refrigeration in Codex. It was adopted by the *Codex Alimentarius* as a guideline in 1991 (9) following an evaluation by JECFA. The Lactoperoxidase System (LP-system) operates by the reactivation of the lactoperoxidase enzyme, which is naturally present in raw milk, by the addition of thiocyanate and a source of peroxide. In milk, the LPs can be bacteriostatic or bactericidal against a diversity of milkborne spoilage and pathogenic bacteria (10, 28, 43, 44, 45)**.** However, *Codex Alimentarius* (8) has identified the need of having greater information about the risk of exacerbation of pathogen bacteria present in milk because of the inhibition of the natural flora present in raw milk.

Consequently, the present study was undertaken to study the effect of the LPs activation on the exacerbation of several potential human pathogens: ***Salmonella spp., Staphylococcus aureus Escherichia coli 0157:H7 and Listeria monocytogenes*** in cow milk activated with the Lactoperoxidase System.

Material and Methods

There was an initial study about milk microbiological quality (total bacteria and somatic cells count, and determination of inhibitor substances). In all cases, milk samples were negative to the pathogens studied. The raw milk, used in two replicas for each microorganism studied, had good quality with an average of 8600 CFU/mL and 450 000 cel/mL. In all the cases, they were free of microbial growth inhibitor substances. Later, the foodborne pathogen: ***Salmonella typhimurium (ATCC 14028), Staphylococcus aureus (ATCC 25923), Escherichia coli enterohemorragic (O157:H7)*** and ***Listeria monocytogenes (ATCC 43256)*** were inoculated. The experiment was carried out at the Microbiology Laboratories of the National Center for Animal and Plant Health (CENSA) and the Food Nutrition and Hygiene Institute in Cuba (INHA) (both accredited by the standard NC/ISO 17025: 2000)(35), using the techniques established for the detection and/or enumeration of the pathogens: ***Staphylococcus aureus*** (ISO 6888-1:1999)(27), ***Salmonella typhimurium*** (ISO 6579, 1993)(25), ***Listeria monocytogenes*** (ISO 11290-1 and 2, 1996)(26) and ***Escherichia coli 0157:H7*** (FDA, 2002)(17). The study carried out for each pathogen in both laboratories (CENSA-INHA), in parallel, with the same milk samples, under the same environment conditions, microorganism strains and analytical diagram.

Contamination was carried out with inocula of $2{,}3x10^5$ CFU/mL for ***Staphylococcus aureus;*** $2{,}1x10^4$ CFU/mL for ***Listeria monocytogenes;*** $2{,}1x10^4$ for ***Salmonella typhimurium*** and $5x10^4$ for ***Escherichia coli 0157:H7,*** respectively. In all cases they were obtained by Mac Farland´s scale and checked by the colony count in Triptycase Soy Agar. Milk preservation temperature for each assay ranged 25 ± 1^0C from 0 to 12 hours. As a carrier of the LPs active principles, a product named STABILAK was used (38), bringing an equivalent quantity of 9 mg/L of sodium thiocyanate and 34 mg/L of sodium percarbonate. Test times per each replica in milk treated with the product (activated milk) and control milk were 0, 4, 8 and 12 hours.

Assay with ***Staphylococcus aureus:***

Dilutions were carried out in the diluter recommended (Triptone C broth) up to 10^{-4}. And later on, 0.1 mL was plated in the surface of Baird Parker agar (two plates for each dilution) of the dilutions 10^{-3} and 10^{-4}. The procedure was repeated passed 4, 8 and 12 hours, increasing a dilution up to 10^{-5}. In the case of the control sample, dilution was increased until 10^{-6}. The plates inoculated were incubated from 24 to 48 hours at 37±1°C. The presumptive colonies of ***Staphylococcus aureus*** were confirmed by coagulase test according to the standard ISO 6888.

Assay with *Listeria* ***monocytogenes:***

Dilutions were carried out in the diluter recommended (Triptone C broth) up to 10^{-3}. And later on, 0.1 mL was plated in the surface of Oxford agar (two plates for each dilution) of the dilutions 10^{-2} and 10^{-3}. The procedure was repeated passed 4, 8 and 12 hours, increasing a dilution up to 10^{-5}. The plates inoculated were incubated from 24 to 48 hours at 37±1°C. The presumptive colonies of ***Listeria monocytogenes*** were confirmed according to the standard ISO 11290-2.

Assay with ***Salmonella typhimurium***:

Dilutions were carried out in the diluter recommended (Triptone C broth) up to 10^{-3}. And later on, 0.1 mL was plated in the surface of Green Brilliant agar (two plates for each dilution) of the dilutions 10^{-2} and 10^{-3}. The procedure was repeated passed 4, 8 and 12 hours, increasing a dilution up to 10^{-4}. The

plates inoculated were incubated at 24 hours at 37±1°C. The presumptive colonies of ***Salmonella typhimurium*** were confirmed according to the standard ISO 6579.

Assay with ***Escherichia coli 0157:H7:***
Dilutions were carried out in the diluter recommended (Triptone C broth) up to 10^{-3}. And later on, 0.1 mL was plated in the surface of Mac Conkey Sorbitol agar (two plates for each dilution) of the dilutions 10^{-2} and 10^{-3}. The procedure was repeated passed 4, 8 and 12 hours, increasing a dilution up to 10^{-4}. The plates inoculated were incubated at 24 hours at 37±1° C. The presumptive colonies of ***Escherichia coli 0157:H7*** were confirmed according to FDA methodology.

There was a CFU/mL count in each assay, according to the technique recommended. These values were transformed to $\log_{10}$. Later, data for 12 hours were processed using t de Student test for two unequal variance samples, so 8 hours was the effective time of the action of the LPs, and 12 hours for evaluating the possible exacerbation of the bacteria studied.

Results

The LPs effects observed at 12 hours, according to *Staphylococcus* ***aureus*** and ***Salmonella typhimurium*** determination***,*** did not show significant differences in the counting in activated milk with respect to the control (Figures 1 and 2), but the control sample showed a higher growth than the activated milk. In the case of *Listeria monocytogenes* (Figure 3) and *Escherichia coli O 157:H7,* (Figure 4), there was a significant reduction ($P<0.05$). The general behaviour evidenced that after 8 hours, when there was not already effective time of the LPs action, there was not a bacterial increase in the activated milk with respect to the control.

Discussion

The results obtained for each pathogen microorganism studied are adjusted to the bacteriostatic/bactericidal effect which has been widely reported in literature (11, 12, 13, 16).

The effect of the LPs obtained in this study against the gram negative bacteria such as ***Salmonella typhimurium*** and ***Escherichia coli*** was not bactericidal as referred in other studies carried out with liquid culture media, ice cream, raw milk, infantile milk formula and a pH in synthetic medium (16, 20, 34, 36, 41). In this experiment, the variations in sensitivity between strains may be explained by different cell-wall structures and inhibitory compounds generated by the organisms concerned. However, the effect obtained against enterohemorrhagic ***E. coli*** is very important, because this microorganism is associated to alimentary intoxications transmitted by milk and dairy products (31).

For ***Staphylococcus aureus***, some studies carried out in raw milk have demonstrated a bactericidal activity at 4 hours (3). Others referred a bacteriostatic activity at 6 hours at 30ºC and at 72 hours at 37ºC with a reduction of 2 log de CFU/mL (30). Other authors pointed out a reduction till 4 log CFU/mL at 12ºC per 8 hours. Besides, it has been pointed out that there is an increase of the thermal treatment effect in the elimination of these microorganisms (19, 30, 36). On the other hand, the action of lactoperoxidase against gram + organisms is generally bacteriostatic and not lethal (14, 29, 32). In our study, the results coincide with the previously referred bacteriostatic effect.

Listeria monocytogenes is one of the most important emerging pathogens reported in alimentary intoxications (18, 21). In our assay, there were significant differences at 12 hours post activation; there was a reduction of a log CFU/mL of the activated milk with respect to the control, observing this behaviour from 4 hours post activation. These results are similar to those referred in other papers, where there is a bacteriostatic effect in milk reducing 3 log CFU/mL at 24 hours at 30ºC (2, 4 ,5). Other

papers pointed out that at refrigeration temperatures, the bacteriostatic effect and the elimination by thermal treatment are increased (22, 37). On the other hand, a bactericidal effect has been obtained at 4ºC, 8ºC, 35ºC and 37ºC at 56 hours (15, 46).

During the past several years, an additional work has been published, especially as related to synergistic effects with other agents and additional information concerning the action of the Lactoperoxidase System against both ***E. coli O157:H7*** and ***Listeria monocytogenes*** (24).

In a general way, though there are some differences among microorganisms, the general behaviour indicates that there was no exacerbation of the pathogens studied at 12 hours after the activation of the LPs. That is why; there is not any microbiological risk for the consumer, associated to the use of this preservation method. If we also add that at this time and in industrial conditions, milk should have been thermally processed, this reduces even more the possibilities of appearing any pathogen in the final product, associated to the use of this preservation method.

References

1. **Aune, T. M., and E. L. Thomas**. 1977. Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. *Eur. J. Biochem*. 80:209-214.
2. **Bibi, W., and M. R. Bachmann.** 1990. Antibacterial effect of the lactoperoxidase-thiocyanate-hydrogen peroxide system on the growth of ***Listeria spp***. in skim milk. *Milchwiss*.45:26-28
3. **Bosch, E. H., Van Doormen, and S. De Vries.** 2000. The lactoperoxidase system: the influence of iodide and the chemical and antimicrobial stability over the period of about 18 months. *J. Appl. Microbiology* 89:2 15-224.
4. **Boussouel, N, F. Mathieu, V. Benoit, M. Linder, A. M. Revel-Junelles, and J. B. Milliere.** 1999. Response surface methodology, an approach to predict the effects of lactoperoxidase system, nisin, alone or in combination, on ***Listeria monocytogenes*** in skim milk. *J. Appl. Microb.* 86:642-652.
5. **Boussouel, N., F. Mathieu, A. M. Revel-Junelles, and J. B. Milliere.** 2000. Effects of combinations of lactoperoxidase system and nisin on the behaviour of ***Listeria monocytogenes*** in skim milk, International *J. Food Microb.* 61: 169-175.
6. **Cailliez-Grimal, C., A. M. Revel-Junelles, M. Linder, and J. B. Milliere.** 2002**.** Antimicrobial activity spectra of the glucose/glucose oxidase and the lactoperoxidase systems (SCN) modified by 1.or 103.anion. *Milchwiss*.57:656-660.
7. **Campbell W.** 2001. Supplemental report in favor of grade raw milk. Disponible in: http://home.earthlink.net/˜optimal/3-8.
8. **Codex Alimentarius** 2005. Text from Report of the 19th Session of the Codex Alimentarius Commission, 1991 regarding limitation in the use of LP-s. Disponible in: http://www.fao.org/docrep/meeting/005/t0490e00.htm
9. **Comisión del Codex Alimentarius, Comité del Codex sobre Leche y Productos Lácteos** 1991**.** Directrices para la conservación de la leche cruda mediante la aplicación del Sistema de la Lactoperoxidasa. CAC/GL 13-1991.
10. **Dennis, F., and J.P. Ramet**. 1989. Antibacterial activity of the lactoperoxidase system on ***Listeria monocytogenes*** in trypticase soy broth, UHT milk and French soft cheese. *J. Food Prot*. 52:706-711.
11. **Dionysius, D. A., P. A. Grieve, and A. C**. **Vos.** 1992. Studies on the lactoperoxidase system: reaction kinetics and antibacterial activity using two methods for hydrogen peroxide generation. *J. Appl. Bacteriology* 72: 146-l 53.
12. **Earnshaw, R. G., J. G. Banks, C. Francotte, and D. Defrise.** 1990. Inhibition of ***Salmonella typhimurium*** and ***Escherichia coli*** *in* enfant formula milk by an activated lactoperoxidase system. *J. Food Prot*. 53: 170-l 72.

13. **El-Agamy, E. I., R. Ruppanner, A. Ismail, C. P. Champagne, and R. Assaf.** 1992. Antibacterial and antiviral activity of camel milk protective proteins. *J. Dairy Res.* 59:169-175.
14. **El-Agamy, E. I., Y. M. R. Shoukry, and M. S. Elghannam.** 1993. Effect of lactoperoxidase activation system on the shelf-life of raw milk stored at different temperatures. Alex. *J. Agricultural Res*.38:35 1-364.
15. **El-Shenawy, M. A., H. S. Garcia, and E. H. Marth.** 1990. Inhibition and inactivation of ***Listeria monocytogenes*** by the lactoperoxidase system in raw milk, buffer or semisynthetic medium. *Milchwiss.*45:638-641.
16. **Farrag, S. A., F. E. El-Gazzar, and E. H. Marth.** 1992. Inactivation of ***Yersinia enterocolitica*** by the lactoperoxidase system in a semi-synthetic medium and in raw milk. *Milchwiss.* 47:95-97.
17. **FDA/ Center for Food & Drug Administration** 2002. Diarrheagenic Escherichia coli. Bacteriological Analytical Manual Online. Chapter 4A. Disponible in: http://www.cfsan.fda.gov/~ebam/bam-4a.html
18. **FDA/Center for Food Safety & Applied Nutrition** 2004. Foodborne Illness Ten Least Wanted Foodborne Pathogens. Hypertext updated by mow/cjm/kwg/dms/ear/dvd/dav.
19. **Francois, M.D** 1998. Enzymes du lait et maitrise du risque sanitaire des produits alimentaires frais et crus. BIO SERAE LABORATOIRES SA. FRANCE: 1-15
20. **García, H.S.; Pardio, V.T.; and K.N. Wauszewski** 1984. Activación del sistema lactoperoxidasa para preservación de leche cruda. *Tecnología Alimentos México*: 18:3:13-18.
21. **Gay, Marielle and A. Amgar** 2005. Factors moderating ***Listeria monocytogenes*** growth in raw milk and in soft cheese made from raw milk. *Lait* 85: 153-170

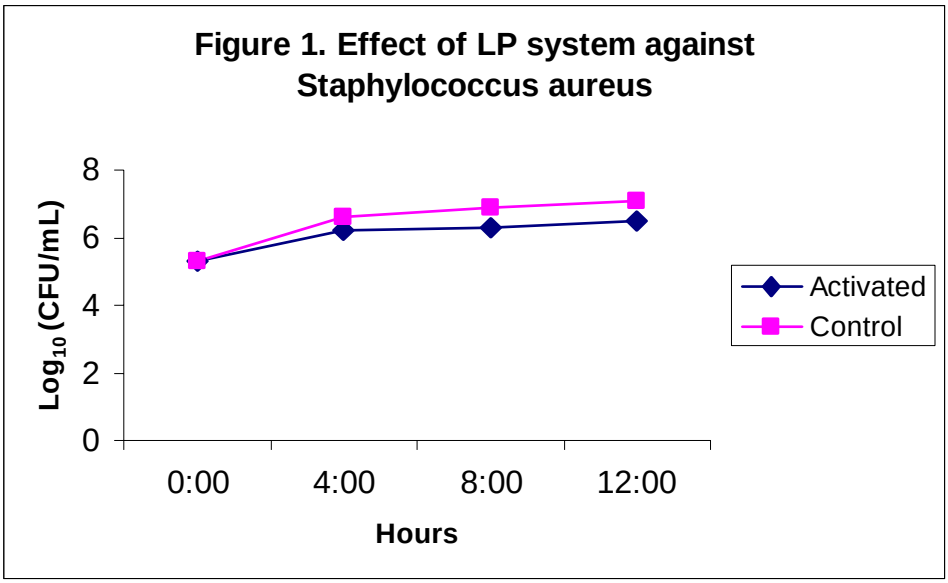
22. **Gaya, P., M. Medina, and M. Nuñez**. 1991. Effect of the lactoperoxidase system on ***Listeria monocytogenes*** behavior in raw milk at refrigeration temperatures. *Appl. Environmental Microb*.57:3355-3360

23. **Gwan C. and C. FaChow** 2000. Studies on the lactoperoxidase system and its use in extending the storage period of cow's raw milk. *J. Chinese Soc. Anim. Sci.* 29(1):89-99.
24. **Harper, W.J and J.T. Parker**. 2000 Biological properties of milk components in Dairy Foods UPDATE IN PROGRESS JUNE 2003. Disponible in: http:// www.fst.ohio-state.edu/People/Harper/Functional-foods/Milk%20Components/Lactoperoxidase.html
25. **ISO 6579** 1993. Microbiology of food and animal feeding stuffs. General guidance on methods for the detection of *Salmonella*.
26. **ISO 11290-1/2** 1996: Microbiology of food and animal feeding stuffs. Horizontal method for the detection and enumeration of ***Listeria monocytogenes.***
27. **ISO 6888** 1999. Microbiology of food and animal feeding stuffs. Horizontal method for the enumeration of coagulase-positive staphylococci (***Staphylococcus aureus*** and other species). Part 1: Technique using Agar Baird Parker medium.
28. **Jacob, B. M., K. Essy Antony, B. Sreekumar, and M. Haridas.** 2000. Thiocyanate: mediated antifungal and antibacterial property of goat milk lactoperoxidase. *Life Sci.* 66:2433-2439.
29. **Kamau D.N., Dooresy S. y Pruitt K.M.** 1990. Antibacterial activity of the lactoperoxidase system against ***Listeria monocytogenes*** and ***Staphylococcus aureus*** in milk. *J. Food Prot.* 53, 1010-1014.
30. **Kangumba, J. G. K., E. H. Venter, and J. A. W. Coetzer.** 1997. The effect of the lactoperoxidase system and souring on certain potential human pathogen in cow's milk. *J. South African Vet. Ass*. 68: 130-136.
31. **Kendall, P.** 2003. Health. Bacterial Food-Borne Illness. *Food and Nutr. series* No.9.300.
32. **Korhonen, K.** 2004. **The lactoperoxidase system in mastitic milk.** FAO Memory LP Expert Meeting 29 February – 1 March 2004.Cape Town, South Africa.

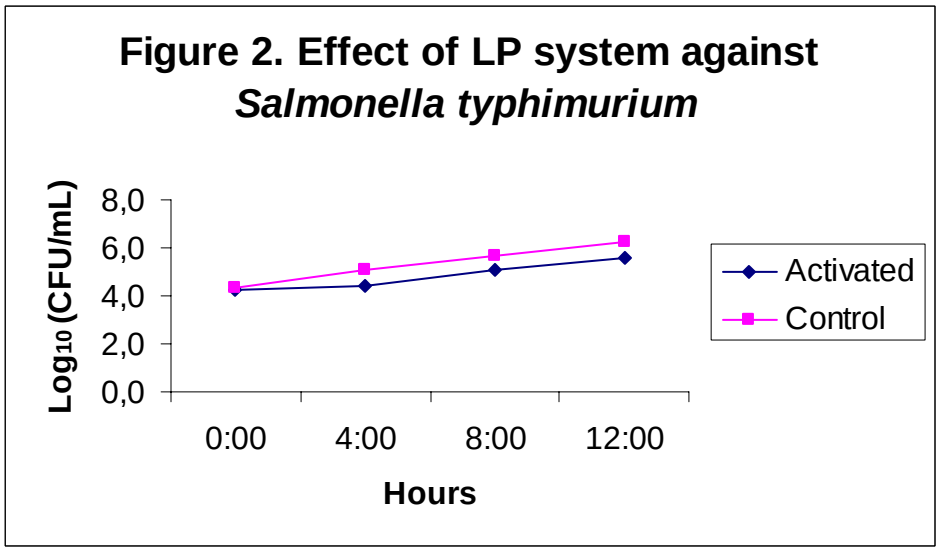
33. **Kussendrager K.D and A.C.M Hooijdank van** 2000. Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. *British J. Nutr.* 84 (Supplement 1): 19-25.
34. **McLay J.C; M.J Kennedy; A.L. Orourke; R.M. Elliot and R.S. Simmonds** 2002. Inhibition of bacterial foodborne pathogens by the lactoperoxidase system in combination with monolaurin. *Int J Food Microbiol*. 25;73 (1):1-9.

35. **NC-ISO/IEC 17025.** 2000. Requisitos Generales para la Competencia de los laboratorios de ensayo y calibración.
36. **Pitt W. M., T. J. Harden and H. R. Hull** 2000**.** Investigation of the antimicrobial activity of raw milk against several foodborne pathogens. *Milchwiss*. 55:249- 252.
37. **Pitt, W. M., T. J. Harden and H. R. Hull.** 1999. Antibacterial activity of raw milk against ***Listeria monocytogenes***. Australian *J. Dairy Techn*.54:90- 93.
38. **Ponce C. P.*, Armenteros A. M.*, Villoch C.*, Montes de Oca N.*, Carreras J.**** 2005**.** Evaluación de riesgos microbiológicos y químicos de la activación del sistema lactoperoxidasa en leche cruda. Disponible in: http://bvs.sld.cu/uats/rtv_files/2005/rtv0505.htm Vol. 9, No. 5 Septiembre-Octubre, 2005 ISSN 1028-4362.
39. **Pruitt, K. M., and B. Reiter**. 1985. Biochemistry of peroxidase system: antimicrobial effects, p. 143-178. In K. M. Pruitt and J.Tenovuo (ed.), The lactoperoxidase system. Marcel Dekker, Inc., New York.
40. **Reiter, B.** 1985. The biological significance of the non-immunoglobulin protective proteins in milk: lysozyme, lactoferrin, lactoperoxidase, p. 281-336. In P. F. Fox (ed.), Developments in dairy chemistry, vol. 3. Elsevier Applied Science Publishers, New York.
41. **Reiter, B.; Marshall. V. and S.M Philipps**. 1976. Nonspecific bactericidal activity of the lactoperoxidase-thiocyanate-hydrogen peroxide system of milk against ***Escherichia coli*** and some gram negative pathogens. *Infect. Immun*: 13 (3):800-807.
42. **Revol-Junelles, A. M., N. Boussouel, J. P. Ramet, and J. B. MilliCre.** 2001**.** Antibacterial activities of the lactoperoxidase systems (LPS) modified by 1.and 10, anions. Milchwiss.56: 329-332.
43. **van Hooijdonk, A. C. M., K. D. Kussendrager, and J. M. Steijns.** 2000**.** *In vivo* antimicrobial and antiviral activity of components in bovine milk and colostrums involved in non-specific defense. *British J. Nutr*. 84: 127-S 134.
44. **Van Opstal, I., Bagamboula, C.F., Vanmuysen, S.C.M., Wuytack, E.Y., and C.W Michiels** 2004**.** Inactivation of *Bacillus cereus* spores in milk by mild pressure and heat treatments. *International J. Food Microb.* 92**:**227-234.
45. **Wolfson, L. M., and S. S. Sumner.** 1993. Antibacterial activity of the lactoperoxidase system: a review. *J. Food Prot*. 56:887-892.
46. **Zapico, P., M. Medina, P. Gaya and M. Núñez.** 1998. Synergistic effect of nisin and lactoperoxidase system on ***Listeria monocytogenes*** in skim milk. *International J. Food Microb*. 40:35-42.

Figures



No significant differences at 12 hours



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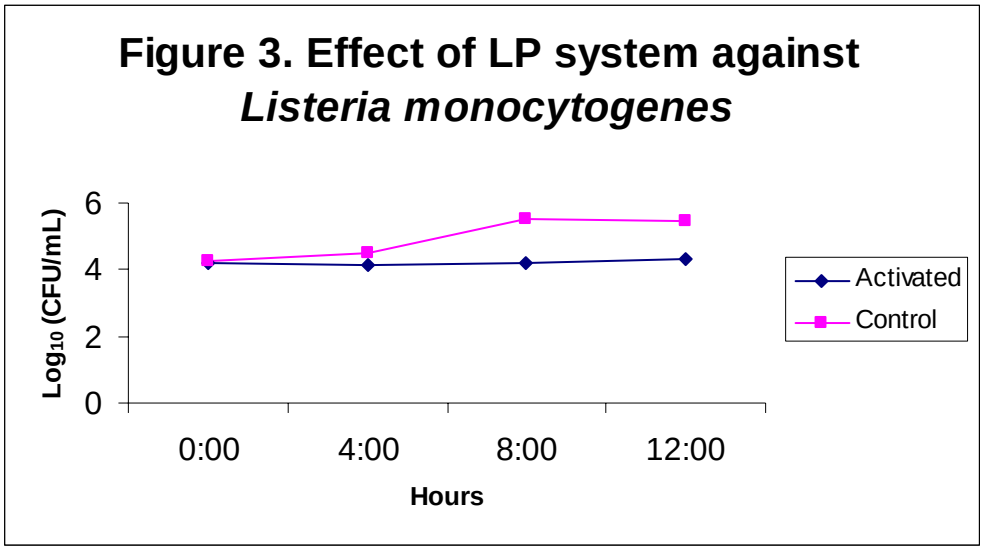
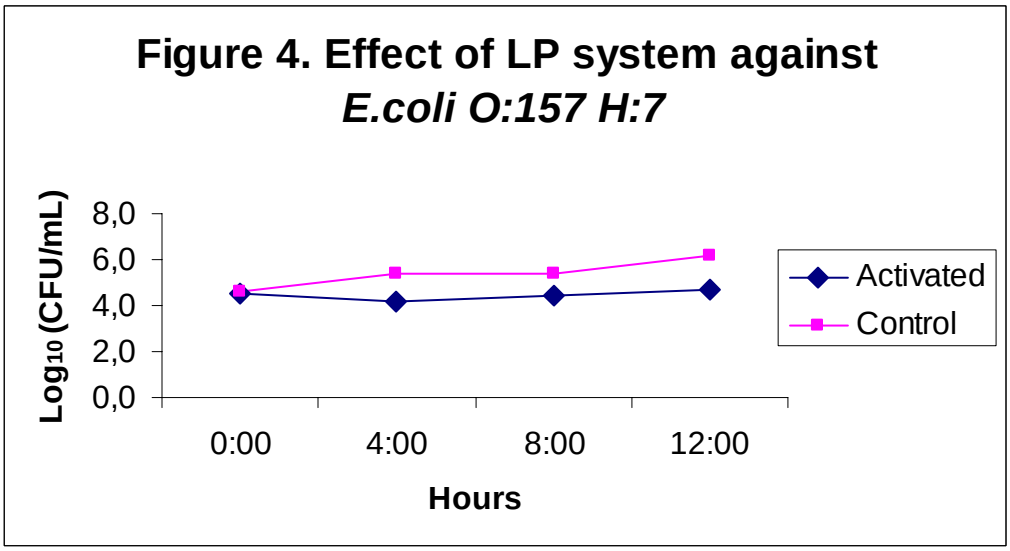


Significant differences P<0.05 at 12 hours



Significant differences P<0.05 at 12 hours