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RISK ANALYSIS OF THE EXACERBATION OF FOODBORNE PATHOGENS IN RAW MILK ACTIVATED WITH THE LACTOPEROXIDASE SYSTEM

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ABSTRACT: **One of the most studied systems in the last 20 years. The lactoperoxidase-thiocyanate-hydrogen peroxide system (LP System) is due to its potential possibilities to avoid raw milk deterioration against undesirable microorganisms. There are experimental and practical evidences demonstrating that the use of this method is innocuous. That is why, the Committee of Experts for Food Additives (JECFA) has pointed out that it is acceptable from the toxicological point of view. 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 several foodborne pathogens: *Salmonella spp.*, *Staphylococcus* coagulase-positive, *Escherichia coli 0157:H7*, *Listeria monocytogenes* and *Bacillus cereus* in raw milk. The experiment was carried out at the Venizie Institute in Italy. The raw milk, used in the three replicas for each microorganism studied, had good quality and it was free of microbial growth inhibitor substances. Later, foodborne pathogens were inoculated with charges 10^2-10^4 CFU/mL. The analyses used were those techniques established for the detection and/or enumeration of these pathogens. As a carrier 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 (activated milk) and control milk were 0, 4, 8 and 12 hours. The LPs effects observed at 12 hours, according to the total bacterial count, showed a highly significant reduction ($P<0.01$) of the counting in activated milk with respect to the control; for *Staphylococcus* coagulase-positive, *Bacillus cereus* and *Listeria monocytogenes*, though there were no significant differences, but the control sample showed a higher growth than the activated milk. In the cases of *Salmonella spp.* and *Escherichia coli O 157:H7*, there was a similar trend between both samples without significant differences. The general behavior indicates that there was no exacerbation of the pathogens studied. Thus 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; milk)

ANÁLISIS DE RIESGO DE LA EXACERBACIÓN DE PATÓGENOS ASOCIADOS A INTOXICACIONES ALIMENTARIAS A PARTIR DE LECHE DE VACA ACTIVADA CON EL SISTEMA LACTOPEROXIDASA

RESUMEN: **El sistema lactoperoxidasa ha sido ampliamente estudiado en los últimos 20 años, debido a las posibilidades potenciales de evitar el deterioro de la calidad de la leche cruda, por los microorganismos contaminantes de la misma. Las evidencias prácticas y experimentales indican que el uso del método es inocuo a la salud humana. En este sentido, el Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) ha señalado que el mismo es aceptable desde el punto de vista toxicológico. Sin embargo, el Codex ha identificado que necesita mayor información en relación con el posible riesgo de exacerbación de las bacterias patógenas, una vez que se debilita el efecto de inhibición de la flora saprófita contaminante natural de la leche. El presente estudio consistió en la evaluación del efecto de la activación**

del sistema LP en la exacerbación de diversas bacterias patógenas: *Salmonella spp., Staphylococcus* coagulasa positiva*, Escherichia coli 0157:H7, Listeria monocytogenes* y *Bacillus cereus.* El experimento se realizó en el instituto IZE de Venecia, en Italia. Se utilizó leche cruda, libre de los patógenos indicados y se sustancias inhibidoras del crecimiento microbiano en tres replicas por cada microorganismo. Dichos patógenos fueron inoculados en cargas de 10^2-10^4 UFC/ml. Los análisis utilizados fueron los establecidos internacionalmente para dichos patógenos. como activador del sistema LP fue utilizado el producto comercial Stabilak, que contiene cantidades equivalentes de 9 mg/L de la sal de tiocianato de sodio y 34 mg/L de percarbonato de sodio. Los tiempos para cada replica de leche tratada y control fueron a 0, 4, 8, y 12 horas. La activación del sistema LP a las 12 horas mostró una reducción altamente significativa ($p<0,01$) en el conteo total de bacterias., No se observó significación entre activado y control para *Staphylococcus* coagulasa positivo, *Bacillus cereus* y *Listeria monocitogenes,* aunque la muestra control siempre mostró mayores crecimiento que en la activada. En el caso de *Salmonella* spp. y *Escherichia coli O157:H7,* se observó una tendencia similar. El comportamiento general indica que no existió exacerbación de los patógenos y que no existen riesgos microbiológicos para los consumidores, asociados al uso del método.

(Palabras clave: sistema LP; bacterias patógenas; análisis riesgo; leche)

INTRODUCTION

Different natural substances and enzymatic and cellular complexes have been identified in milk. They have a proved antibacterial capacity; some of them of a specific immunologic nature and some others of unspecific one. One of the most studied systems in the last 20 years is the lactoperoxidase-thiocyanate-hydrogen peroxide system (LP System) due to its potential possibilities to avoid raw milk deterioration against undesirable microorganisms. The presence of LPs components has been recognized in the mammary gland, and even in the thin intestine of young animals.

The activation of the LPs has been conceived as a mean for preventing deterioration of raw milk by the action of bacteria during its obtainment, collecting and transport, till its processing (8). The principle of use is guided towards the bacteria which cause acidification and other alterations in the physic-chemical characteristics, and no specifically as a way for reducing foodborne pathogens. Due to its antimicrobial nature, it does not exclude the activity against them. Guidelines clearly establish the need of keeping the Good Hygiene Practices and also the processing of raw material, clearing up that the LPs does not guarantee inocuity of milk and dairy products by itself. Its antimicrobial effect has been widely demonstrated, behaving as a bactericidal/bacteriostatic agent against different groups of microorganisms such as pathogen bacteria, viruses, mycoplasmas, yeasts, fungi, parasites (6,1923,30,32).

There are experimental and practical evidences demonstrating that the use of this method is innocuous. That is why, the Committee of Experts for Food Additives (JECFA) has pointed out that it is acceptable from the toxicological point of view. 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 several foodborne pathogens: *Salmonella spp., Staphylococcus* psi*, Escherichia coli 0157:H7, Listeria monocytogenes* and *Bacillus cereus* in raw milk.

MATERIAL AND METHODS

There was an initial study about milk quality from the microbiological point of view (total bacterial count, somatic cells and determination of inhibitor substances). The raw milk, used in the three replicas for each microorganism studied, had good quality with an average of 7000 CFU/mL and 247 000 cel/mL. In all the cases, they were free of microbial growth inhibitor substances. Later, the foodborne pathogen *Salmonella spp.* (ATCC 14028), *Staphylococcus* coagulase-positive (ATCC 25923), *Escherichia coli O157:H7* and *Listeria monocytogenes* (ATCC 43256) were inoculated. The experiment was carried out at the Venizie Institute in Italy. The analyses were based on IZS analytical requirements and procedures of

Venizie, accredited by the register 0155, using the techniques established for the detection and/or enumeration of the pathogens: *Staphylococcus* coagulase-positive ((ISO 6888-1:1999), *Salmonella spp.* (ISO 6579, 1993), *Listeria monocytogenes* (ISO 11290-1 y 2, 1998) and *Escherichia coli* 0157:H7 (*Metodología FDA-BAM, Método C, modificado 1995)*.

Contamination was carried out with charges 10^2-10^4 CFU/mL, obtained by Mac Farland´s scale and checked by the countings made in Triptycase Soy Agar. Milk preservation temperature for each assay ranged from 21-27,4^{0}C from 0 to 12 hours. As a carrier of the LPs active principles, a product named STABILAK was used (26), 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.

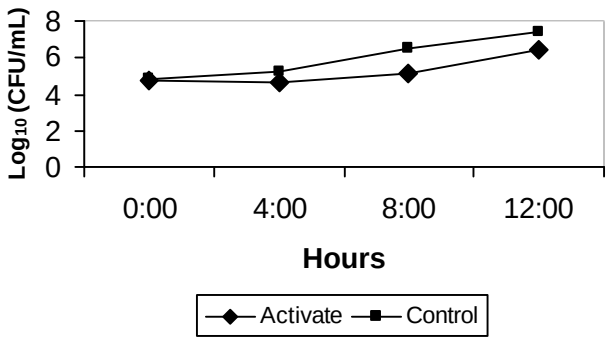
There was a CFU/mL or MPN/mL counting in each assay, according to the technique recommended. These values were transformed to $\log_{10}$. Later, data for 12 hours were processed using t de Student test for two unequal variance samples, so an 8 hour period was the effective time for the action of the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

RESULTS

The LPs effects observed at 12 hours, according to the total bacterial count, showed a highly significant reduction (P<0.01) of the counting in activated milk with respect to the control (Figure 1); for *Staphylococcus* positive coagulase (Figure 2), *Bacillus cereus* (Figure 3) and *Listeria monocytogenes,* (Figure 4), though there were no significant differences, but the control sample showed a higher growth than the activated milk. In the cases of *Salmonella spp.* (Figure 5) and *Escherichia coli O 157:H7* (Figure 6), there was a similar trend between both samples without significant differences. The general behavior evidenced that after 8 hours, when there was not already effective time of the action of the LPs, there was not a bacterial increase in the activated milk with respect to the control.

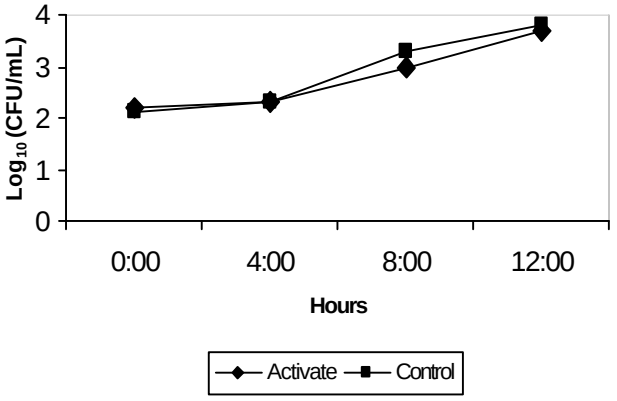
DISCUSSION

The results obtained for each pathogen microorganism studied are adjusted to the bacteriostatic/bactericidal effect which has been widely reported in literature (9, 10, 11, 14, and 20).



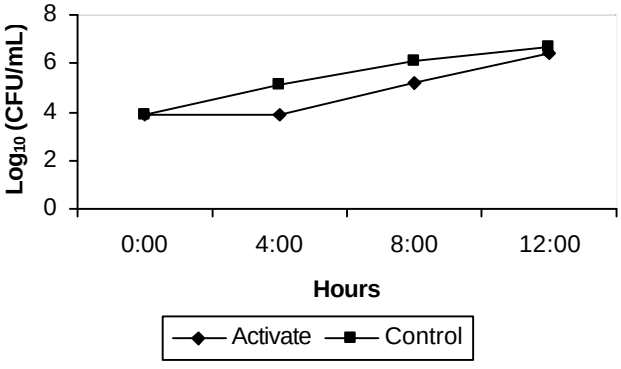
Significant differences P<0.01 at 12 hours.

FIGURE 1. Effect of LP system against total bacterial count./ *Efecto del sistema LP frente al conteo toal de basterias.*



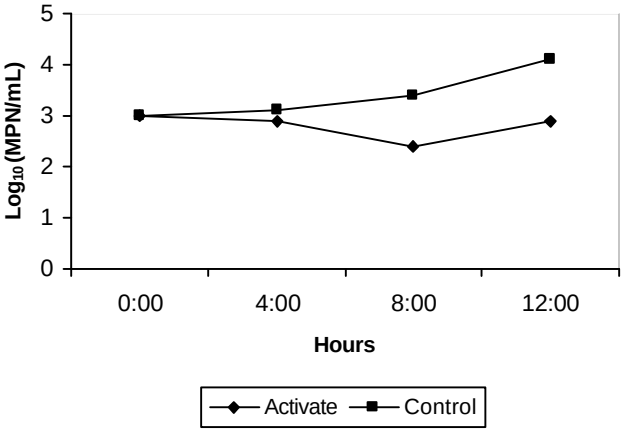
No significant differences at 12 hours.

FIGURE 2. Effect of LP system against Staphylococcus coagulase positive./ *Efecto del sistema LP frente a Staphylococcus coagulase-positive.*



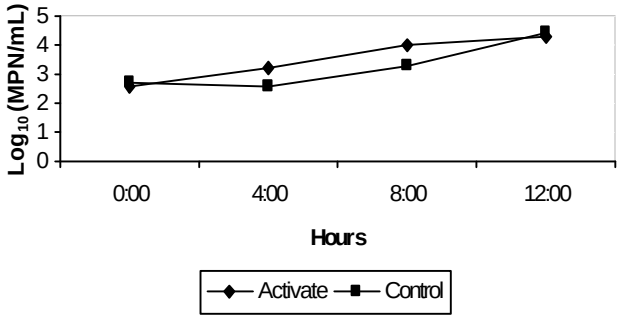
No significant differences at 12 hours.

FIGURE 3. Effect of LP system against ***Bacillus cereus***./ *Efecto del sistema LP frente a* ***Bacillus cereus****.*



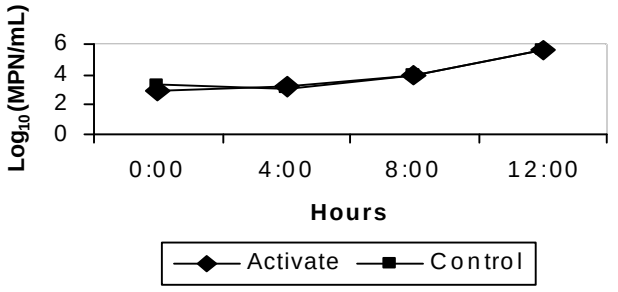
No significant differences at 12 hours.

FIGURE 4. Effect of LP system against ***Listeria monocytogenes***./ *Efecto del sistema LP frente a* ***Listeria monocytogenes.***



No significant differences at 12 hours.

FIGURE 5. Effect of LP system against *Salmonella* spp./ *Efecto del sistema LP frente a* ***Salmonella*** *spp.*



No significant differences at 12 hours.

FIGURE 6. Effect of LP system against ***E.coli O:157 H:7***./ *Efecto del sistema LP frente a* ***E. coli O:157 H:7.***

The results obtained for the total bacterial count coincide with other studies, where a bacteriostatic effect with a reduction of 2 and 3 log de CFU/mL has been observed in raw milk at 8 hours after activating the system (1,11,16).

The effect of the LPs obtained in this experiment against the gram negative bacteria studied such as *Salmonella spp.* and *Escherichia coli* was not bactericidal as referred in other studies carried out with liquid culture medium, ice cream, raw milk, infantile milk formula and a pH in synthetic medium (17,24, 28); though it should taken into account that the response observed in this experiment could have influenced in the high pathogenicity in the strains used.

For *Staphylococcus* coagulase-positive, 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 (12, 20). 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 (16, 21, 25). 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. In our case, it was demonstrated that in spite of not having significant differences at 12 hours of activation, there was a reduction of a log CFU/mL of the activated milk with respect to the control, observing this behavior from 4 hours of the 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 (18, 24). On the other hand, a bactericidal effect has been obtained at 4ºC, 8ºC, 35ºC and 37ºC at 56 hours (13,33).

The results obtained with *Bacillus cereus*, which is a microorganism used in this study as an indicator for evaluating the effect of the LPs against spore-formers, showed that if there was no a significant difference at 12 hours of activation between milk treated with the product and the control from 4 to 8 hours, there was a reduction of a log of CFU/mL. This response allows confirming that, in the case of spoilage microorganisms; there is not a possible exacerbation in the activated milk. On the other hand, there are references of a bactericidal/bacteriostatic activity of this system in milk against this type of microorganisms (12, 29, 31).

In a general way, though there are some differences by microorganisms, the general behavior 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 under 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.

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