

Full Length Research Paper

Optimizing antimicrobial activity of the bovine lactoperoxidase system against *Salmonella enterica* Hadar, a causative agent of human gastroenteritis in Tunisia

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In this work, it was shown that activating the Lactoperoxidase system (LP system) has an antimicrobial effect against *Salmonella enterica* serovar Hadar (*S. enterica* Hadar), a foodborne pathogen widespread in Tunisia and therefore a major cause of gastroenteritis. The aim here was to optimize the concentrations of the various compounds used in activating the LP system and to test the efficiency of this optimized system on *S. enterica* Hadar growth. It was also demonstrated that using a single dose of activators (thiocyanate and percarbonate) was enough to obtain the antimicrobial effect of this system against *S. enterica* Hadar. Since lactoperoxidase has a broad antimicrobial spectrum, it can be used as an additional interesting way to improve food security in Tunisia and reduce the use of chemicals. It is possible to combine the LP system with bioactive molecules or introduce other non-thermal technologies for the bio-preservation of food. Within this context, it was shown that the antimicrobial effect of the LP system is inhibited in the presence of a concentration of 100 g / L of starch. The starch content in food should be taken into consideration when the LP system is applied in synergy with other natural conservation techniques.

Key words: Lactoperoxidase, raw cow milk, *Salmonella enterica* Hadar, starch, keeping quality.

INTRODUCTION

Salmonella is a ubiquitous enteric bacterium. It is one of the most common causes of bacterial gastroenteritis. *Salmonella* outbreaks have generally been associated with consumption of contaminated food or water (Threlfall, 2002). Each year, nearly 2000 strains of *Salmonella* are reported throughout Tunisia by the National Center for Enteropathogenic Bacteria of the Pasteur Institute (Centre National de *Salmonella*, *Shigella*, et *Vibrio* – Institut pasteur Tunisia) (Abbassi-Ghozzi I et al., 2012). According to Issa et al. (2007), *Salmonella enterica* serovar Hadar (*S. enterica* Hadar) belongs to the list of the largest 15 sero-

types in Tunisia. It showed strong peaks scattered throughout the 11 year study period. It can be isolated from milk and dairy products. *S. enterica* Hadar has a wide range of animal reservoirs.

Bioactive peptides derived from bovine milk have been shown to exert beneficial effects on human health. These biological properties may play an important role in the development of medical foods that treat or mitigate the effects of diseases. With the rise of consumer concerns about the deleterious effects of chemical preservatives and an increasing preference for natural components,

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milk-derived bioactive peptide may be valuable in food preservation (Pepe et al., 2013).

This work, focuses on the Lactoperoxidase system (LP system), a native antimicrobial system of milk. The LP system, consisting of lactoperoxidase, thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2) (Fweja et al., 2007), is an antimicrobial system that occurs naturally in milk and other secretions such as saliva and tears (Singh et al., 2012; Wijkstrom-Frei et al., 2003). Lactoperoxidase is one of the most stable enzymes of milk (Griffiths, 1986) and its inactivation is used as a good pasteurization indicator (Barrett et al., 1999). Lactoperoxidase catalyzes the oxidation of thiocyanate by H_2O_2 into short-lived intermediate products with antibacterial properties, such as hypothiocyanite (OSCN^-) anion (Nagy et al., 2009, Pruitt et al., 1982). These reactive products oxidize sulphhydryl groups (SH) of proteins in the bacterial cell membrane and inhibit several important enzymes in cellular metabolism (Shin et al., 2001; Kussendrager et al., 2000).

The use of the LP system has been promoted as an effective method of extending the shelf-life of raw milk in developing countries because it provides natural short-term preservation. The use of the LP system, combined with good hygiene, is a reliable and economical method of preserving milk, where refrigeration is not possible (CAC 1991). The LP system can be used in combination with other non-thermal conservation techniques such as high hydrostatic pressure and pulsed electric fields (Garcia-Graells et al., 2000) or in synergy with other bioactive molecules (Shin et al., 2012; Arques et al., 2008).

The goal here is to use the LP system along with those techniques which do not require heating or chemical preservatives in order to maintain flavor and nutritional value. However, protein, fat, or other components in complex food substrates may protect target microorganisms, for instance, by adsorbing antimicrobial components (Garcia-Graells et al., 2000). The work was divided into two phases. During the first phase, the effects were tested for the first time of antimicrobial activity of the LP system against *S. enterica* Hadar, a pathogen causing gastroenteritis in Tunisia (CNR-Slam, 2007). During the second phase, and in order to use the LP system in synergy with other non-thermal preservation techniques to prepare milk-derived foods, the effects were studied of a concentration of 100 g / L of starch on the antimicrobial activity of the LP system.

MATERIALS AND METHODS

The bacterial strain

S. enterica subsp. *enterica* serovar Hadar (*Salmonella* Hadar, isolate 63) (6, 8: Z10: e, n, X) was isolated at the Pasteur Institute of Tunis, Tunisia, (CNR-Slam, 2007).

Source of milk

Raw milk samples were obtained from a milk collection center in Mrezig EL BADER Group in the region of Bizerte.

Study and optimization of the LP system activation on the growth of *S. enterica* Hadar

Two Erlenmeyer flasks containing 10 ml of raw milk each were inoculated with *S. enterica* Hadar to a final concentration of 58×10^5 CFU/ml (Touch et al., 2004). A single or double dose of activators was added to each flask. According to the original guidelines of the Codex Alimentarius Commission of 1991 (CAC 1991), an activator dose is 14 mg/L of sodium thiocyanate (NaSCN) and 30 mg/L of sodium percarbonate ($\text{Na}_2\text{CO}_3 \cdot 3\text{H}_2\text{O}_2$). Thiocyanate ions (SCN^-) and hydrogen peroxide (H_2O_2) are activators of the lactoperoxidase naturally occurring in the milk, but at low concentrations. By adding an appropriate amount of activators, the lactoperoxidase can be reactivated in raw milk to inhibit the growth of germs. An Erlenmeyer flask containing raw milk inoculated with *S. enterica* Hadar without activating the LP system was used as a negative control. All treatments, including controls, were performed in triplicate. The preparations were incubated at 25°C for 12 h. During this period, the LP system remained active (CAC 1991). Enumeration of *S. enterica* Hadar was done every four hours on an SS medium (Difco) (ISO, 2007).

Study of the effect of a concentration of 100 g/L of starch on the antimicrobial activity of the LP system against *Salmonella enterica* Hadar

10 ml of raw milk containing activators were inoculated with *S. enterica* Hadar to a final concentration of 58×10^5 CFU/ml and 100 g/L of starch (concentration generally used in the industry to prepare foods made from milk). Two controls were prepared. The first control contained raw milk inoculated with *S. enterica* Hadar containing an activator. The second control was raw milk inoculated with *S. enterica* Hadar without activating the LP system where 100 g/L of starch was added. All treatments, including controls, were performed in triplicate. The preparations were incubated at 25°C for 12 h. Enumeration of *S. enterica* Hadar was done every 4 h on an SS medium.

Statistical analysis

All experiments were performed three times. Data were analyzed by ANOVA (STATISTICA version 6.0 for Microsoft Windows), and middle pairs were compared with the (HSD) Tukey test. Significant differences were defined at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

RESULTS AND DISCUSSION

This project focused on the LP system, a natural system for conserving raw milk. The work was divided into two main steps: The first phase was to optimize the LP system action. This was done by using different doses of thiocyanate and percarbonate, two activators of the system. We tested this optimization on the growth of a foodborne pathogen, *S. enterica* Hadar. The results presented in Figure 1 shows that after 12 h of growth in the LP system activation with a single dose of activator,

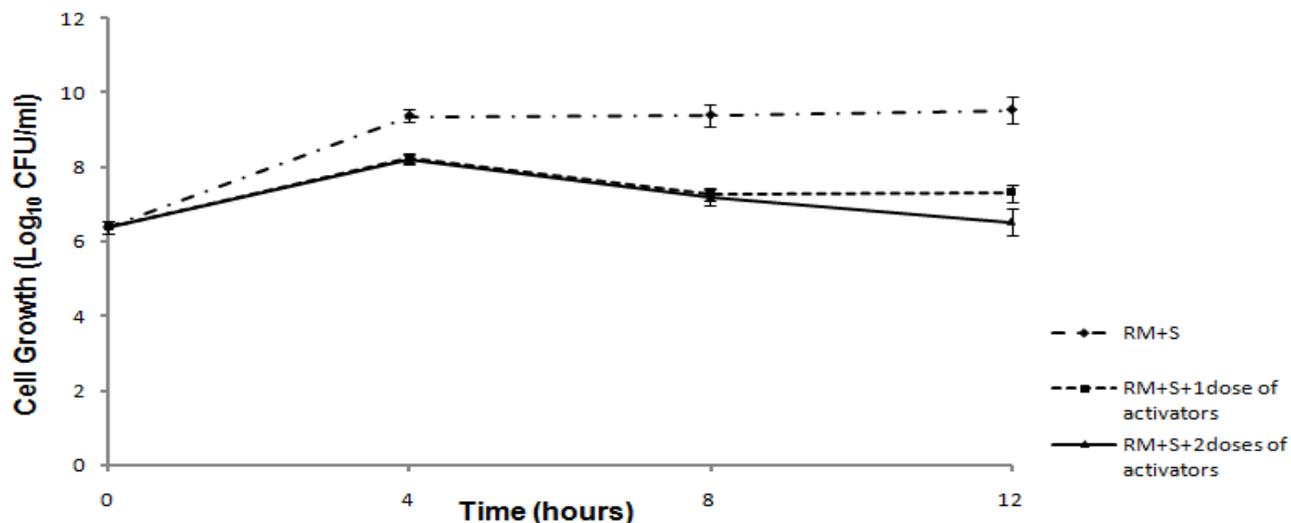


Figure 1. Efficacy of the lactoperoxidase system in inhibiting the growth of *Salmonella enterica* Hadar in raw milk. RM, Raw milk; S, *Salmonella enterica* Hadar; 1 dose of activator = 14 mg/L of sodium thiocyanate and 30 mg/L of sodium percarbonate. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

Table 1. Efficacy of the lactoperoxidase system in inhibiting the growth of *S. enterica* Hadar in raw milk.

Doses of activator	Number of <i>S. enterica</i> Hadar (Log ₁₀ UFC/ml) at the initial time (t = 0 min)	Number of <i>S. enterica</i> Hadar (Log ₁₀ UFC/ml) after 12 hours (t = 12 h)
Without activator	6.39	9.53
Single dose	6.39	7.31
Double doses	6.39	6.53

1 dose of activator = 14 mg/L of sodium thiocyanate and 30 mg/L of sodium percarbonate.

the number of *S. enterica* Hadar rose from 6.39 to 7.31 log₁₀ CFU/ml compared to 9.53 log₁₀ CFU / ml for the control. After activating of the LP system with a double dose, the number of *S. enterica* Hadar rose from 6.39 to 6.53 log₁₀ CFU/ml (Table 1). According to these results, it was concluded that *S. enterica* serotype Hadar was sensitive to the antimicrobial effect of the LP system. After 12 h of growth with a single dose of activator, the results obtained showed a significant decrease (**p < 0.001) in the number of *S. enterica* Hadar compared to the control. The effect of using a double dose of activator was not highly significant (*p<0.05) compared to the use of a single dose after 12 h. A single dose was sufficient to activate the antibacterial effect against *S. enterica* Hadar, without excessively increasing the concentration of activators in raw milk.

In the second phase, in thinking of applying this system to the preservation of foods prepared from milk, the effects were studied of a concentration of 100 g/L of starch on the antimicrobial activity of LP system against *S. enterica* Hadar. After 12 h of incubation in the presence of 100 g/L of starch and after activating the LP

system in raw milk, the number of *S. enterica* Hadar increased 6.44 to 8.00 log₁₀ CFU/ml compared to 10.27 log₁₀ CFU/ml in the absence of activating the LP system (Table 2). Based on these results, it was observed that, even in the presence of a high concentration of starch, the antibacterial effect of the LP system remained highly significant (**p<0.001). However, after 12 h of growth after activating the LP system and in the absence of starch, 7.18 log₁₀ CFU/ml (**p<0.001) was found. According to these results, it was concluded that the effectiveness of the antimicrobial effect of the LP system dropped 20% in the presence of a concentration of 100 g/L of starch.

Many studies have been devoted to the lactoperoxidase system in various countries. It has been proven that the system inhibits the growth and proliferation of several germs such as *S. enterica* (Touch et al., 2004), *Escherichia coli* O157:H7 (Parry-Hanson et al., 2009), *Staphylococcus aureus* and *Candida albicans* (Sisecioglu et al., 2010). According to the work of Garcia-Graells et al. (2000), there is a fundamental difference between bacterial species and sub-species in their response to the

Table 2. Effect of a concentration of 100 g/L starch on the antimicrobial activity of LP system against *S. enterica* Hadar.

Parameter	Number of <i>S. enterica</i> Hadar (Log ₁₀ UFC/ml) at the initial time (t = 0 min)	Number of <i>S. enterica</i> Hadar (Log ₁₀ UFC/ml) after 12 hours (t = 12 h)
RM+S+Sta	6.44	10.27
RM+S+LPS	6.44	7.18
RM+S+LPS+Sta	6.44	8.00

RM, Raw milk; S, *Salmonella enterica* Hadar; Sta., starch (100 g/L); LPS, activation LP system with 1 dose of activator (14 mg/L of sodium thiocyanate and 30 mg/L of sodium percarbonate).

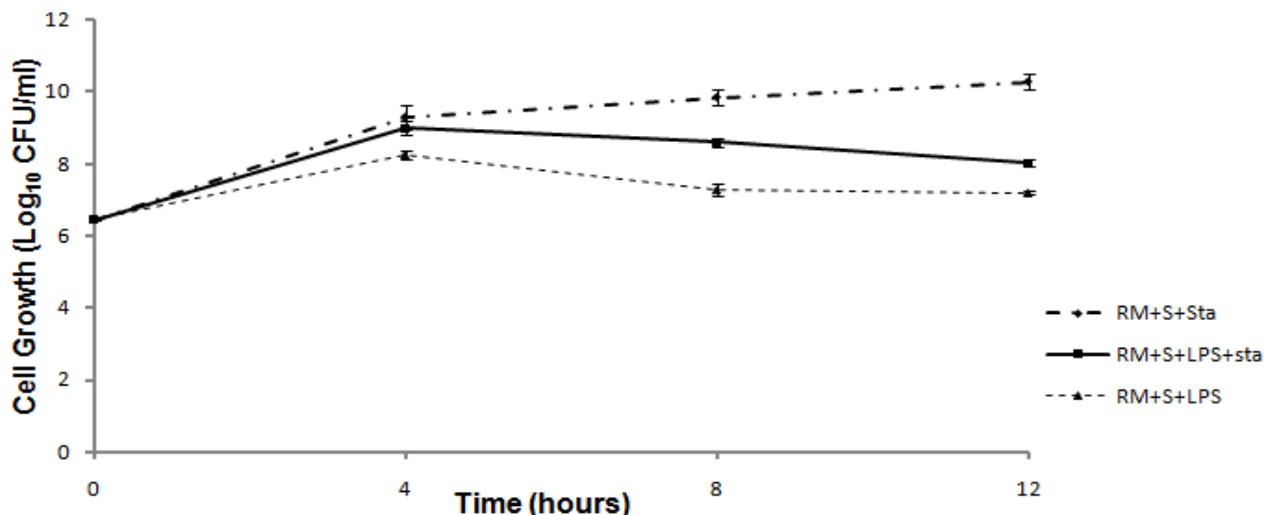


Figure 2. Effect of concentration of 100 g/L of starch on the antibacterial activity of the LP system. RM, Raw milk; S, *Salmonella enterica* Hadar; Sta, starch (100 g/L); LPS, activation the LP system with 1 dose of activator (14 mg/L of sodium thiocyanate and 30 mg/L of sodium percarbonate). Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

LP system. Therefore, interest lies in the study of the antibacterial effect of the LP system against *S. enterica* serotype Hadar.

According to Figures 1 and 2, we see that the antimicrobial effect of the LP system against *S. enterica* Hadar begins after 4 h of growth. In fact, 4 h is the time required for the thiocyanate ions to cross the cytoplasmic membrane and pass into the cytoplasm to inhibit bacterial enzymes (Touch et al., 2004). Moreover, studies have shown that treatments aimed at weakening the bacterial cell envelope increase the effectiveness of the antimicrobial activity of the LP system (Thomas, 1981). The LP system has been recognized as essential in the dairy industry for preserving raw milk (Haddadin et al., 1996), cheese (Earnshaw et al., 1989), yogurt (Hirano et al., 1998) and pasteurized milk (Marks et al., 2001). The field of conservation through the LP system has been extended to make it possible to conserve powdered milk for infants (Oshima et al., 2012) and bleaching whey (Campbell et al., 2012).

In Tunisia, the percentage of *Salmonella* isolation demonstrated a marked pattern of seasonality, increasing in the warm spring months for food and animal isolates (Ben Issa et al., 2007). This observation emphasizes the importance of hygiene and food processing and handling measures in breaking the chain of transmitting *Salmonella* from animal to food. Applying the LP system in Tunisia may have beneficial effects on the treatment of raw milk and pasteurized milk (Barrett et al., 1999) by extending their shelf life.

This system can also be used in the future for processing and producing other dairy products. Improving food safety could be an interesting additional antibacterial obstacle in combination with other non-thermal techniques or bioactive molecules. Within this context, it is interesting to see the effects of starch content on applying the LP system for food conservation. According to the results shown in Figure 2, a decrease was observed of the antimicrobial effect of the LP system in the presence of a concentration of 100 g/L of starch. This can be ex-

plained by the fact that starch is a carbon source for *Salmonella*, thus promoting its growth. In addition, studies have shown that the catalytic activity of lactoperoxidase decreased in the presence of sugars (Al-Baarri et al., 2011). In these conditions, a loss of thiocyanate ions was seen as a result of absorption in food emulsions.

For the first time in Tunisia, this work has made it possible to study the effects of antimicrobial activity of the LP system against *S. enterica* Hadar, a causative agent of acute gastroenteritis. Results clearly show that the serotype Hadar is sensitive to the antimicrobial activity of lactoperoxidase in the presence of a single dose of activator. The LP system could be an effective means of bio-preservative against *S. enterica* Hadar in certain foods, especially those made from raw milk.

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