Antibacterial effect of the lactoperoxidase system against Aeromonas hydrophila and psychrotrophs during the manufacturing of the Spanish sheep fresh cheese Villalón

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1. Introduction

The lactoperoxidase-thiocyanate-hydrogen peroxide system (LP system) of milk is one of the better known natural antimicrobial systems of food (12). Its bacteriostatic and bactericidal activity against several foodborne bacteria has been previously shown (17). Also, the activation of the LP system in raw milk has been proposed as a means for controlling several sanitary risks of bacterial origin and for improving its shelf-life (9).

Aeromonas hydrophila is a Gram-negative bacterium with increasing concern as a food-borne pathogen (8, 15). Virulent *Aeromonas* strains were detected in soft cheeses ready for consumption (13). This kind of food is common in the diet of people included in the epidemiological groups being highly susceptible to foodborne infections (children, elderly). In Spain, Villalón cheese is one of the most consumed soft fresh cheeses. It is made of ewes' milk, and it is characterized by its high moisture content, high pH and low salt level (3). The antibacterial effect of the LP system against several *Aeromonas* isolates of dairy origin in broth and ewes' milk has been previously demonstrated (14). However, the antibacterial activity of the LP system depends, among other factors, on the substrate where the assays are carried out (17). On the other hand, the enzymatic activity of psychrotrophic bacteria from raw milk is one of the main factors affecting the shelf-life of fresh cheeses (4).

The purpose of this work was to investigate the activity of the LP system against *A. hydrophila*, as well as against psychrotrophs during Villalón cheesemaking.

2. Materials and methods

2.1 Microorganisms

The type strain of *A. hydrophila* (NCTC 8049) was used. This strain was initially isolated from a tin of milk with a fishy odour (National Culture Type Collection Catalogue). The strain was kept on Tryptone Soya Agar (Unipath, Basingstoke, England) slants at 4 °C. Before being used in cheese experiments, it was grown twice in Brain Heart Infusion broth (Unipath) at 30 °C/24 h. The second broth was diluted in sterile peptone water (Unipath; 0.1%) in order to prepare the inoculum to be added to pasteurized milk (which was calculated using the McFarland scale).

2.2 Milk

Raw bulk ewes' milk was obtained from a dairy factory in León. Four-litre batches were aseptically collected and immediately transported at refrigeration temperature (4 °C) to our laboratory. SCN^- concentration in raw milk was determined by the method described by BANKS and BOARD (1).

2.3 Cheese-making

Four-litre batches of milk (in Erlenmeyer flaks of 10 l) were pasteurized (63 °C/30 min) in a water bath. The lactoperoxidase activity of pasteurized milk was tested by the reaction with p-phenylene diamine (7).

The pasteurized milk was divided into 2 equal fractions (a and b) which were tempered at 37 °C and then processed following the below scheme:

- Fraction a: After addition of $CaCl_2$ (Panreac, Barcelona, Spain; 0.1% final concentration) it was inoculated with ca. 10^2 cfu/ml of *Aeromonas hydrophila.*
- Fraction b: After addition of $CaCl_2$ (0.1% final concentration) and ca. 10^2 cfu/ml of *Aeromonas hydrophila*, the native LP system was activated by adding KSCN (Sigma Chemical Co., St. Louis, MO, USA; to 25 mM) and H_2O_2 (Panreac; 25 mM).

Each fraction was clotted with calf rennet (Productos Nievi, Bilbao, Spain) in about 45 min. The curd was slightly cut (2 knives cutting in a cross shape), and the whey was allowed to drain for 30 min (at room temperature). Drained curd was manually introduced into cylindrical molds, slightly hand-pressed, and salted for 2 h in a 14.5 °Bé brine. Salted curds were stored at refrigeration temperature (4 °C, 95 % RH) for 48 h. Three batches of cheese were made.

2.4 Microbiological analysis

Samples for microbiological analysis (50 g each) were taken from: curd immediately after draining, cheese after salting, and after 24 and 48 h of refrigerated storage. They were homogenized in sterile 0.1% peptone water (Unipath) with 1% of Tween 80 (Panreac), filtered through a sterile cotton cloth and ten-fold diluted in sterile 0.1% peptone water (Unipath) when needed. *Aeromonas* counts were carried out by spreading 0.1 ml of the appropriate dilution onto Starch Ampicillin Agar plates followed by incubation at 30 °C/24 h (11). Psychrotrophic counts were performed according to APHA (6).

2.5 Physico-chemical determinations

The pH of curd/cheese was measured in every sampling step with a pH-meter fitted with a punction electrode (Crison Instruments, Barcelona, Spain).

Contents of moisture (IDF standard 4:1958), protein (IDF standard 25:1964) and fat (IDF standard 5A: 1969) were determined in each sample of cheese ready for consumption (after 48 h of refrigerated storage).

2.6 Statistical analysis

Microbiologial and physico-chemical data were submitted to a variance analysis using a SPSS PC+ software package (version 3.1, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

The evolution of *Aeromonas* and psychrotrophic populations, as well as pH in curd/cheese during the manufacture of one of the batches of Villalón cheese are shown in Fig. 1. Figures for these parameters in the 2 other batches were similar.

The activation of the LP system of pasteurized milk was bactericidal for *A. hydrophila* during cheesemaking. Fig. 1 shows that, just after salting, *A. hydrophila* was not detectable by the plating method used. In this regard, the bactericidal effect of the LP system against *A. hydrophila* during cheesemaking was similar to that observed with several *A. hydrophila* strains in culture media and in pasteurized ewes' milk at several temperatures (14).

Furthermore, psychrotrophic counts decreased steadily during manufacturing and refrigerated storage of LP-activated cheeses (Fig. 1). EARNSHAW *et al.* (5) reported a complete disappearance of inoculated *Pseudomonas*, *Salmonella* and *Escherichia coli* during cheese-making of Cottage cheese when the LP system was activated. It should be noted that the pH values of the Cottage cheeses were considerably lower than those of the Villalón cheeses (<5.0 *vs.* >6.1), and this fact might have contributed to the antimicrobial effect observed.

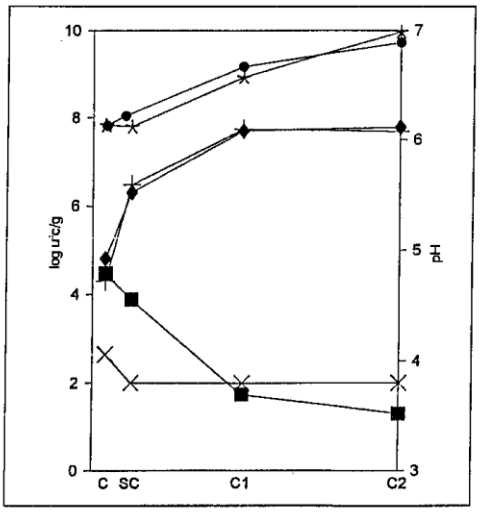

Fig. 1: Effect of the lactoperoxidase system on counts of A. hydrophila and psychrotrophs during manufacturing and refrigerated storage of Villalón cheese. ◆: Psychrotrophic counts in control cheese; +: A. hydrophila counts in control cheese; ■: Psychrotrophic counts in cheese made from LP activated pasteurized ewes' milk (LP-active cheese); X: A. hydrophila counts in LP-active cheese (<2 log cfu/g is represented as 2 log cfu/g); ●: pH in control cheese; ∗: pH in LP-active cheese; C: Curd; SC: Curd immediately after salting; C1: Cheese after 24 h of refrigerated storage (4 °C); C2: Cheese after 48 h of refrigerated storage (4 °C)

Previous works, with *Pseudomonas fluorescens* as the test organism, found that the activation of the LP system in milk caused both an initial bactericidal effect and a later delay of growth of survivors, with both effects depending, among other factors, on the temperature of storage (2, 16). In contrast, the antimicrobial effect of the LP-system against psychrotrophs was stronger in our cheeses than those previously shown (psychrotrophic counts in 48 h cheeses were more than 6 log cfu/g lower in LP-activated cheeses than in control cheeses, Fig. 1). The 3 dominant taxonomic groups of psychrotrophic bacteria found by us in Villalón cheese are: *Enterobacteriaceae*, *Pseudomonas* and *Leuconostoc* (13).

The LP-activated cheeses did not show significant differences in compositional parameters (dry matter, protein and fat) in comparison with the controls. LARA *et al.* (10), in a study carried out on "Mexican-style" fresh cheese, found differences between control and LP-active samples (with highest yields in the latter ones), but the differences could be explained taking into account that they incubated the milk before cheese-making at 28 °C for 4 h, with the initial microbial contamination being very high, so differences in proteolysis and lipolysis could affect the subsequently obtained yields.

4. Conclusion

The activation of the LP system during cheesemaking seems to be a reliable tool for controlling cold tolerant spoilage bacteria, as well as one of the emerging foodborne psychrotrophic pathogenic bacterium (*A. hydrophila*), thus being a useful method (complementary to refrigeration) to extend shelf-life and to improve the bacterial safety of fresh cheeses.

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5. References

(1) BANKS, J.G., BOARD, R.G.: Lett. Appl. Microbiol. **1** 81-85 (1985)
(2) BJÖRCK, L.: J. Dairy Res. **45** 109-118 (1978)
(3) CHAVARRI, F.J., NUÑEZ, J.A., BAUTISTA, L., NUÑEZ, M.: J. Food Protect. **48** 865-869 (1985)
(4) COUSIN, M.A.: J. Food Protect. **45** 172-207 (1982)
(5) EARNSHAW, R.G., BANKS, J.G., DEFRISE, D., FRANCOTTE, C.: Food Microbiol. **6** 285-288 (1989)
(6) FRANK, J.F., CHRISTEN, G.L., BULLERMAN, L. B.: In Standard methods for the examination of dairy products (Ed. R.T. Marshall). Am. Publ. Health Assoc., Washington D.C. (1992)
(7) GUHA, K.C., ROY, B.R.: J. Sci. Food Agric. **24** 1-6 (1973)
(8) JANDA, J.M.: Clin.Microbiol.Rev. **4** 397-410 (1991)
(9) KORHONEN, H.: World Anim. Rev. **35** 23-29 (1980)
(10) LARA, R., MENDOZA, A., DE LA CRUZ, I., GARCIA, H.S.: Milchwissenschaft **42** 773-775 (1987)

(11) PALUMBO, S.A., MAXIMO, F., WILLIAMS, A.C., BUCHANAN, R.L., THAYER, D.W.: Appl. Environ. Microbiol. **50** 1027-1030 (1985)
(12) REITER, B., HÄRNULV, G.: J. Food Protect. **47** 724-732 (1984)
(13) SANTOS, J.A.: Ph. D. Thesis. Univ. León, Spain (1994)
(14) SANTOS, J.A., GONZALEZ, C., GARCIA-LOPEZ, M.L., GARCIA-FERNANDEZ, M.C., OTERO, A.: Lett. Appl. Microbiol. **19** 161-164 (1994)
(15) STELMA, G.N.: In Foodborne Bacterial Pathogens (Ed. M.P. Doyle). Marcel Dekker, Inc., New York (1989)
(16) UCEDA, R., GUILLEN, A.M., GAYA, P., MEDINA, M., NUÑEZ, M.: Milchwissenschaft **49** 139-143 (1994)
(17) WOLFSON, L.M., SUMNER, S.S.: J. Food Protect. **56** 887-892 (1993)

6. Summary

SANTOS, J.A., LÓPEZ-DÍAZ, T.M., GARCÍA-FERNÁNDEZ, M.C., GARCÍA-LÓPEZ, M.L., OTERO, A.: **Antibacterial effect of the lactoperoxidase system against Aeromonas hydrophila and psychrotrophs during the manufacturing of the Spanish fresh cheese Villalón.** Milchwissenschaft **50** (12) 690–692 (1995).

86 Lactoperoxidase system (Aeromonas)

The activation of the lactoperoxidase (LP) system in pasteurized ewes' milk used for making a variety of fresh cheese (Villalón) resulted in a total inhibition of *Aeromonas hydrophila*, when it was added at 10^2 cfu/ml of pasteurized milk, from the end of salting. Also, psychrotrophic populations in cheeses were clearly reduced (more than 6 log cfu/g) by the activation of the LP system in pasteurized milk. However, chemical composition of cheeses (dry matter, protein and fat) was not affected by the activation of the LP system. The activation of the LP system in pasteurized ewes' milk used for manufacturing of fresh cheeses seems to be a useful method for controlling the undesirable effects associated with cold tolerant microorganisms.

SANTOS, J.A., LÓPEZ-DÍAZ, T.M., GARCÍA-FERNÁNDEZ, M.C., GARCÍA-LÓPEZ, M.L., OTERO, A.: **Antibakterielle Wirkung des Laktoperoxidasesystems gegenüber Aeromonas hydrophila und psychrotrophen Keimen während der Herstellung des spanischen Schaf-Frischkäses Villalón.** Milchwissenschaft **50** (12) 690–692 (1995).

86 Laktoperoxidase-System (Aeromonas)

Die Aktivierung des Laktoperoxidase (LP)-Systems in pasteurisierter Schafmilch, die zur Herstellung eines Frischkäses (Villalón) verwendet wurde, ergab eine vollständige Hemmung von *Aeromonas hydrophila*, wenn 10^2 KbE/ml der pasteurisierten Milch nach dem Ende des Salzens zugefügt wurde. Auch waren die Psychrotrophenzahlen in Käsen durch die Aktivierung des LP-Systems in pasteurisierter Milch deutlich reduziert (>6 log KbE/g). Die chemische Zusammensetzung der Käse (Trokkenmasse, Protein und Fett) wurde durch die Aktivierung des LP-Systems nicht beeinflußt. Die Aktivierung des LP-Systems in pasteurisierter Schafmilch erscheint bei der Herstellung von Frischkäsen ein nützliches Verfahren zur Überwachung unerwünschter Effekte, die mit kältetoleranten Mikroorganismen verbunden sind, zu sein.