Inhibition of *Staphylococcus aureus* Growth by Lactic Acid Bacteria in Milk

Bettache Guessas, Miloud Hadadji, Noureddine Saidi and Mebrouk Kihal*

**ABSTRACT**

Lactic Acid Bacteria (LAB) from raw goat and ewes' milk, from arid and semi-arid zones in west Algeria were tested for the production of antimicrobial substances against *Staphylococcus aureus* in milk. Twenty samples of raw milk were collected from which 96 LAB strains were isolated and exhibited antagonistic effects on solid agar medium. Two strains exhibited inhibition towards *S. aureus* from their supernatant culture medium. A *Lactococcus lactis* subsp *lactis* biovar diacetylactis (Lc8) strain proved by far the most efficient, produced a heat stable bactericidal substance with a proteinaceous nature, suggesting a bacteriocin. Lc8 inhibited *S. aureus* in milk, so that no cell was counted after 48h of incubation. This strain acts either by decreasing the pH or by bacteriocin production which makes it a good candidate strain in cheese making and useful in preventing growth of *S. aureus*.

**KEYWORDS:** Lactic acid bacteria, bacteriocin, *Staphylococcus aureus*, milk, bactericidal, *Lactococcus lactis*.

**INTRODUCTION**

In a variety of ecological niches, micro-organisms compete with each other for survival and through evolution form unique flora. In some food ecosystems, Lactic Acid Bacteria (LAB) constitute the dominant microflora. These organisms are able to produce antimicrobial compounds against competing flora, including food-borne spoilage and pathogenic bacteria (Daeshel, 1989; Davidson and Hoover, 1993). Lactic acid bacteria provide a major preservative effect in food fermentations which mankind has practiced for thousands of years. The primary antimicrobial effect exerted by LAB is the production of lactic acid and the reduction of pH (Daeschel, 1989). In addition, LAB produce various antimicrobial compounds, which can be classified as low molecular weight compounds such as hydrogen peroxide, carbon dioxide, diacetyl and high molecular weight compounds like bacteriocins (Piard and Desmazeaud, 1991; 1992; Ouwehand, 1998).

These antimicrobial substances have the potential to inhibit the growth of a narrow range of lactic acid bacteria (Tagg et al., 1976), as well as Gram-positive pathogenic and spoilage bacteria (Klaenhammer, 1988; Cintas et al., 1995; Casla et al., 1996; Enan et al., 1996; Contreras et al., 1997). Some authors have reported their ability also to inhibit Gram-negative species (Blackburn et al., 1989; Lewus et al., 1991; Stevens et al., 1991; Kalchayand et al., 1992; Arihara et al., 1996). The mode of action of some bacteriocins has been identified, but for many others only a general description of the bacteriostatic (Lewus et al., 1991; Thompson et al., 1996) or bactericidal effects (van Belkum et al., 1991; Venema et al., 1993; Schved et al., 1993; Samelis et al., 1994; Enan et al., 1996) has been reported.

In the arid zones of Algeria, the milk preservation factories are unavailable, hence most raw goat’s or ewes’ milk is made into cheese, using goat’s rennet for coagulation. This type of cheese is subject to spoilage especially by pathogenic bacteria like *Staphylococcus aureus*. If milking goat and ewes have mastitis, then large numbers of infectious micro-organisms may be shed in their milk (De Vries, 1975) and consequently form a significant part of the flora of raw milk products such as farmhouse cheeses.

The aim of this work was to isolate bacteriocin-producing LAB capable of inhibiting *Staphylococcus aureus* in order to use them as starters for traditional production of cheese in arid and semi-arid zones.

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* Laboratoire de Microbiologie Appliqué, Département de Biologie, Faculté des Sciences, Université d’Oran, Oran, Algeria. Received on 11/7/2004 and Accepted for Publication on 7/6/2005.
Materials and Methods

Bacterial Strains and Cultures

*Staphylococcus aureus* and *Lactobacillus curvatus* LTH1432 (WP Hammes) was chosen as indicator strains to demonstrate and measure bacteriocin activity. All cultures were maintained as frozen stocks held at -20°C MRS broth with 25% (v/v) glycerol (Difco Laboratories, Detroit, MI, USA). Before experimental use, cultures were propagated twice in broth for 18 to 24 h. The following strains were grown in the indicated media: lactobacilli, pediococci, and enterococci, in MRS (de Man, Rogosa, and Sharp) broth (Difco); and lactococci, in M17 (Difco); and *Staphylococcus aureus* in Tryptic Soy Broth (TSB, Difco).

Isolation and Identification of Antistaphylococcal LAB and Activity Assays

The search for anti-*S. aureus* LAB was carried out by using raw milk as a source of this bacteria. A total of 12 samples of raw goats and ewes’ milk were aseptically collected from different farms in west Algerian arid zones (Table 1) and stored frozen at -20°C with glycerol (10%) until use. Samples were thawed and kept at 25 °C for 4h. Ten fold dilutions of milk samples were spread out on the surface of four MRS agar (0.1 ml of appropriate dilution is used). Plates were incubated anaerobically at 30 °C for 2 days and then overlaid with 5 ml of Tryptic Soy Broth with 0.75% agar which was previously inoculated with 0.1 ml of 18 h culture of *Staphylococcus aureus* in TSB. After overnight incubation, bacterial colonies found positive for anti-staphylococcal activity were identified by their inhibition zones, streaked for single colony isolation, and tested further (from each sample, ten colonies were kept from higher dilution). Isolates were inoculated in MRS broth to yield an initial concentration of ca. 10^3 cfu/ml and incubated for 18 h at 37 °C. After centrifugation (2700Xg for 10 min), the pH of the supernatant was adjusted to 6 with 0.1 M NaOH and filter sterilized (0.45-µm pore size). Antistaphylococcal activity of the obtained cell-free filtrate (culture extract) was tested by the well diffusion assay (deferred method) described by Tagg and McGiven (1971), with double layer consisting of MRS agar and Chapman agar seeded with *Staphylococcus aureus* as for the direct method. To quantitate inhibitory activity, the diameter of the inhibition zone (in millimetres) was measured.

All isolates were tested for their Gram reaction, catalase activity using H_2O_2, shape by observation of overnight cultures using a phase contrast microscope standard and presumptively classified as lactic acid bacteria. Full identification was carried as described in Guessas and Kihal (2004).

Determination of Bacteriocin Activity

Bacteriocin activity was determined by an agar well diffusion assay, as described by Ryan et al. (1996). Molten agar at 48°C was seeded with the indicator strain *Lactobacillus curvatus* (50 µl of an overnight culture per 20 ml agar), dispensed into sterile Petri dishes, and allowed to solidify. Wells of approximately 4-6 mm in diameter were made and bottom selleed by one drop sterile agar. Fifty µl aliquots of two fold serial dilution of bacteriocin preparation were dispensed into the wells. After incubation overnight at 30 °C, bacteriocin activity was calculated as the inverse of the latest dilution that gave a definite zone of clearance. Activity units were expressed per millilitre (1/dilution x 20).

Sensibility to Heat and Proteolytic Enzymes

The culture extract of the selected strain was treated with several enzymes: trypsin; α-amylase and catalase (Sigma Chemical Co., St. Louis, MO, USA). All samples were adjusted to pH 7 with 1.0 M NaOH, filter sterilized (0.45 millipore size), and held for 1 h at 25 °C. The residual activity of the treated and control samples was determined by measuring the diameters of the inhibition zones in the well diffusion assay. To determine the heat sensitivity, cell-free supernatants were heated at 80, 100, 110, and 120 °C each for 10, 15 and 30 min; they were then cooled and tested for residual activity by the agar well diffusion assay.

Growth of *S. aureus* Strain in the Presence of a Bacteriocin Producer

To demonstrate the effect of bacteriocin-producing strain on a growing culture of *Staphylococcus aureus* strain, this bacterium was grown together with the strain Lc8 in reconstituted milk. Selected LAB strains Lc8, and *S. aureus* were grown in skimmed milk (100g/l) for 24 h. Reconstituted milk was divided into 10 ml portions, placed into 45 flasks and heat treated at 80°C for 10 min. Each 15 flasks were inoculated with a 3% of a 24-h culture consisting of Lc8 strain, *Staphylococcus aureus*, the mixed culture of Staph. and Lc8 respectively. The flasks were finally incubated at 30 °C. In order to
estimate growth in single and mixed cultures, variation in cell density of *Staphylococcus aureus* and Lc8 was determined by plating the samples onto specified agar plates medium (Chapman and MRS respectively); in mixed cultures, however only *Staphylococcus aureus* count was made in single cultures.

**Lactic Acid Production**

The lactic acid production by the screened strains of LAB was determined by measuring the pH expressed as Dornic degree (°D) in heat treated (5 min at 121°C) reconstituted skimmed milk (100 g/L). Three percent of 24-h pre-cultured milk was added, and the pH was measured after incubation at 30°C.

**RESULTS AND DISCUSSION**

The inactivation of pathogenic and food spoilage micro-organisms is a major concern with respect to food processing. The food-borne pathogens which are of particular importance in dairy foods include *Staphylococcus aureus* and *Listeria monocytogenes*. *S. aureus* is one of the most problematic microorganisms present in raw milk. If milking goat and ewes have mastitis, then large numbers of infectious microorganisms may be shed in milk (De Vries, 1975) and consequently form a significant part of the flora of raw milk products such as farmhouse cheeses.

A total of 96 bacterial isolates with antagonistic effects on *S. aureus* were isolated from 12 raw goat and ewes milk samples. In most cases, the antagonistic effect was due to a decrease in the pH resulting from the production of organic acids. The culture extracts from two strains were shown to be active against *Staphylococcus aureus* by the action of antibacterial substances other than organic acids. These bacterial isolates were *Lactococcus lactis* subsp lactis (Lc7) and *Lactococcus lactis* subsp lactis biovar diacetylactis (Lc8) (Figures 1,2). The latter proved to be by far the most efficient, with a culture extract activity of 14,025 AU/ml (arbitrary units).

Similar results were obtained by Geis et al. (1983), who tested 93 strains *Lactococcus lactis* subsp. *Cremonis* and found that 36 strains exhibited antagonistic effects on agar, but only one of them produced an antibacterial substance in the liquid medium. Also, Schillinger and Lucke (1989) found only one strain exhibiting antibacterial action in liquid medium from 221 isolates.

Noonpakdee et al. (2003), isolated 14020 lactic acid bacteria isolates in which only one strain exhibited inhibition toward *Staphylococcus aureus*. The Lc8 strain showed a high capability to inhibit *S. aureus* growth. Vaughan et al. (1994), demonstrate the ability of LAB isolated from raw milk to inhibit *S. aureus*.

The antibacterial substances contained in the culture extract of Lc8 strain were not inactivated in the presence of catalase, which exclude an inhibition by hydrogen peroxide. They were however inactivated by the proteolytic enzyme trypsin, and no change in their activity was observed when they were treated with α-amylase. In fact, the initial culture extract produced an inhibition zone with a diameter of 10 mm in *Staphylococcus aureus* culture. Later, no inhibition zone was detected after treatment with these enzymes (Figure 3). The antibacterial substance is, therefore, a substance of a proteinaceous nature. Since no change was observed upon treatment with α-amylase, it can be inferred that no carbohydrate moiety is essential for the bacteriocin activity (Jack and Jung, 2000). To evaluate the heat stability of these antistaphylococcus substances, cell-free supernatants were incubated at 80, 100, 110, and 120 °C for various periods of time. There was no remarkable reduction in the antibacterial activity after heating at 80°C for 30 min.

The addition of the concentrated neutralized culture supernatant of Lc8 to a culture of *S. aureus* in TSB broth resulted in a rapid inactivation of the sensitive cells. The number of viable cells per millilitre declined from 1.5x10⁶ to 1.2x10⁴ after 8 hours of incubation (Figure 4) and below 500 cells per ml after 24 hours, and cells did not regrow within 48 h. In contrast, the culture of *S. aureus* was unaffected by the addition of a concentrated supernatant of the bacteriocin-negative strain. These results indicate a bactericidal mode of action of the antibacterial compound. The bactericidal mode of action and the proteinaceous nature of this substance are typical characteristics of bacteriocin (Tagg et al., 1976). Similar results were reported by Galvin et al. (1999) who found that *Lactococcus lactis* subsp. *lactis* DPC3147 is active against *S. aureus* and reduced considerably cell numbers after 2 h in time-kill curve conducted in broth medium.

The acidity produced by Lc8 strain in skimmed milk evaluated by the amount of lactic acid released and expressed as Dornic degree show a production of 17 °D lactic acid after 6 h and 35 °D after 8 h of culture. The production increased to reach 40 °D after 24 h of culture.
The final pH is about 4.64 at 8 h and about 4.36 after 24 h of incubation.

The inhibition of a growing culture of *S. aureus* by adding a bacteriocin-producing strain Lc8 in milk was examined and compared with the growth of *Staphylococcus aureus* in milk (figure 5). A decrease in *S. aureus* count after 8 h was noted; on the other hand, in single culture, the growth of *S. aureus* increased after the same period of time. After 24 h, the decrease in *S. aureus* growth was considerable and continued until only two bacteria were counted after 48 h.

We note the double action of Lc8 either by acidifying action and/or a bacteriocin production. The acidity is the first step in cheese making to reach suitable pH to form a curd. Acidity and bacteriocin production together play an important role in cheese making as described by Ryan *et al.* (1996). The inhibition of *S. aureus* by Lc8 strain is due to bacteriocin production, however the acidity plays a combined role in this inhibition. Similar results were reported by Noonpkdee *et al.* (2003) who isolated about 14020 strains from fermented sausage and found that only one strain was capable of reducing pH in a manner similar to our finding.

**Table 1: Type and source of raw milk sample used in this study.**

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