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Acidification is not involved in the early inhibition of *Staphylococcus aureus* growth by *Lactococcus lactis* in milk

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Abstract

Seventy-five *Lactococcus lactis* strains were screened for their inhibitory effect on *Staphylococcus aureus* growth in milk. Most lactococcal strains had a strong antagonistic effect. Characterization of this effect showed that acidification was not involved in the inhibition observed within the first 24 h of mixed culture. Alternate effects such as bacteriocin- or hydrogen peroxide-production were eliminated. These results question some generally accepted ideas and show that even low acidifying *L. lactis* strains, widely used in raw milk soft cheeses, can efficiently inhibit *S. aureus* growth even with initial contamination levels as high as 10^3 cfu mL^{-1}.

Keywords: *Staphylococcus aureus*; *Lactococcus lactis*; Mixed cultures; Growth inhibition; Milk

1. Introduction

Starter lactic acid bacteria (LAB) diversity is used in combination with different process technologies to offer a wide variety of fermented dairy products. Growth and activity of LAB also have an inhibitory effect on spoiling and pathogenic bacteria (Nes & Johnsborg, 2004; Rossland, Langsrud, Granum, & Sorhaug, 2005) including enterotoxin-producing strains of *Staphylococcus aureus*, a Gram-positive pathogen frequently involved in food poisoning outbreaks (Le Loir, Baron, & Gautier, 2003). In many countries, some cheeses are still made with raw milk. These traditional cheese-making processes are important to maintain agricultural activity in areas unfavorable to intensive agriculture, frequently associated with the development of organic agriculture and considered as a real gastronomic patrimony. However, they have to face the social demand on food safety leading to severe sanitary criteria and tougher rules. *S. aureus* is a frequent causative agent of mastitis. It is thus a frequent raw milk contaminant and may grow during the cheese-making process (De Buyser, Dufour, Maire, & Lafarge, 2001; Delbes, Alomar, Chougui, Martin, & Montel, 2006; Jorgensen, Mork, & Rorvik, 2005). Enterotoxin production in foodstuffs occurs (or is detectable) when enterotoxigenic *S. aureus* population reaches $\sim 10^6$ cfu mL^{-1} (Le Loir et al., 2003). Thus, it is quite interesting to select starter LAB able to efficiently inhibit *S. aureus* growth. LAB–*S. aureus* interactions have been explored for years (Ammor, Tauveron, Dufour, & Chevallier, 2006; Haines & Harmon, 1973b; Kao & Frazier, 1966; Otero & Nader-Macias, 2005; Schellenberg, Smoragiewicz, & Karska-Wysocki, 2006). Several parameters were proposed as involved in *S. aureus* inhibition by LAB, including bacteriocin- (Ammor et al., 2006) and hydrogen peroxide-production (Otero & Nader-Macias, 2005), competition for nutrients (Haines & Harmon, 1973a) and, of course, acidification (Barber & Deibel, 1972; Delbes et al., 2006; Lindqvist, Sylven, & Vagsholm, 2002; Notermans & Heuvelman, 1983). Concerning acidification, several studies have established a direct relationship between pH and level of growth by and survival of *S. aureus* in model media (Iandolo, Ordal, & Witter, 1964; Minor & Marth, 1970; Notermans & Heuvelman, 1983) or in various fermented products (Delbes et al., 2006; Metaxopoulos, Genigeorgis, Fanelli, Franti, & Cosma, 1981; Olarte, Sanz, Gonzalez-Fandos, & Torre, 2000). A great variability was found in

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the inhibitory activity among complex starter LAB mixtures and among LAB species (Haines & Harmon, 1973a; Kao & Frazier, 1966) but only two reports estimated the inhibitory efficacy at the intra-species level, one of which included *Lactococcus lactis*, the model LAB and one of the most-used LAB species in cheese production (Haines & Harmon, 1973b). This study suggested that bacteriocin-independent inhibition is similar among strains within the *L. lactis* species. Here, a large-scale analysis of *L. lactis* antagonistic potential against *S. aureus* clearly questions the homogeneity of the antagonistic potential within *L. lactis* species as well as the importance of acidification in the early inhibition of *S. aureus* growth by *L. lactis* in milk.

2. Material and methods

2.1. Bacterial strains and growth conditions

The 75 *L. lactis* strains screened in this study came from the CNRZ collection of INRA (UMR STLO INRA Agrocampus Rennes, France) and included 36 *L. lactis* subsp. *lactis* strains (48%), 19 *L. lactis* subsp. *lactis biovar diacetylactis* strains (25%), and 20 *L. lactis* subsp. *cremoris* (27%). These *L. lactis* strains were used in fermented dairy products (in the name of the strains, IL stands for Industrie Laitière). Two *S. aureus* strains were used in this study: *S. aureus* LM48, a staphylococcal enterotoxin A-producing strain isolated from cow milk, in 2002, in a French dairy farm (laboratory collection, Rennes, France) and *S. aureus* RN4220, a laboratory strain (Peng, Novick, Kreiswirth, Kornblum, & Schlievert, 1988). *S. aureus* RN4220 is a well-characterized and transformable *agr*-deficient derivative of an *S. aureus* strain isolated from a human infection.

Mixed cultures with *S. aureus* and *L. lactis* strains and pure cultures of *S. aureus* strains (used as control) were grown in tubes at 30 °C, under static conditions, on M17 broth containing 0.5% lactose (AES, Combourg, France) or low heat skim milk (kindly provided by P. Schuck, UMR STLO INRA Agrocampus Rennes, France) reconstituted in sterile water (10% (w/v); hereafter referred to as LH milk). The same stock of LH milk powder was used for the entire study to avoid any variability in milk composition. LH milk properties are similar to raw milk ones, ensuring mixed culture conditions close to traditional cheese-making process (Garem, Schuck, & Maubois, 2000). Media were inoculated using pre-cultures grown overnight under static conditions on the same medium at 25 °C (*L. lactis*) or 37 °C (*S. aureus*). For cultures on LH milk, two successive subcultures in milk were grown to allow adaptation to the medium. Predetermined volumes of each pre-culture were added to mixed and control cultures to obtain initial cell concentrations of 10^3 and 10^6 cfu mL^{-1} for *S. aureus* and *L. lactis*, respectively. Those concentrations were determined according to field conditions. For *L. lactis*, a population of 10^6 cfu mL^{-1} corresponds to the concentration used for starter LAB in cheese-making process. For *S. aureus*, a population of 10^3 cfu g^{-1} of end product corresponds to the level of contamination tolerated in some raw milk cheeses.

Cultures in M17 broth or LH milk at constant pH were grown in 2 L fermentor (Setric Génie Industriel, Toulouse, France). Regulation of pH at the initial value (i.e. 6.8 for milk and 6.9 for M17) was achieved by automatic addition of NaOH (5 M).

2.2. Analytical methods

Bacterial growth was followed by CFU determination using the micromethod previously described (Baron et al., 2006). *S. aureus* population was determined on Tryptic Soy Broth (TSA, AES, Combourg, France) agar plates supplemented with 6.5% NaCl and incubated 24 h at 37 °C. Total population was determined on M17 agar plates incubated 24 h at 30 °C. The results reported here are the mean counts of at least two independent experiments.

Lactic acid concentration was measured by high-pressure liquid chromatography (HPLC). Prior to analysis, proteins were precipitated by adding 0.02 M H_2SO_4 and one drop of HCl (37%) to 2 mL of milk culture sample. After centrifugation (10000 × *g*, 15 min, 4 °C), the supernatant was filtered through a 0.2 μm Millipore filter. Separation was achieved using an Aminex A.6 column (Biorad, Marnes la Coquette, France) and 0.02 M H_2SO_4 as eluent at a flow rate of 1 mL min^{-1}. The column effluents were monitored using an UV spectrophotometric detector at 210 nm (Michalski et al., 2004).

2.3. Effect of pH/lactic acid on S. aureus growth in LH milk

Cultures were grown as described above, except that 0–1% lactic acid was added prior to inoculation, which resulted in milk acidification (pH 6.8–4.1). The effect of lactic acid (0–4%) on *S. aureus* growth in skim reconstituted milk was also investigated at pH 6.8 after neutralization of pH by addition of NaOH (10 M).

2.4. Detection of bacteriocin-producing strains

Bacteriocin production was detected as described previously (Piard, Delorme, Giraffa, Commissaire, & Desmazeaud, 1990). Tested *L. lactis* strains were grown on M17 medium until late exponential phase. A 10 μL sample of culture supernatant was then laid, after neutralization by NaOH, on a M17 agar plate seeded with *L. lactis* CNRZ117, a strain known to be sensitive to most bacteriocins. Plates were incubated at 30 °C for 24 h to allow growth of *L. lactis* CNRZ117. The presence of bacteriocin in the tested supernatant led to an inhibition zone, due to the diffusion of the bacteriocin through the agar. *L. lactis* CNRZ481, a bacteriocin-producing strain, was used as a positive control. Experiments were repeated twice. Similar experiments were performed using *S. aureus* RN4220 and LM48 as test strains.