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Inhibition of *Clostridium tyrobutyricum* in cheese by *Lactobacillus gasseri*

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Abstract

A semi-hard cheese produced from milk artificially contaminated with *Clostridium tyrobutyricum* spores ($2.5 \times 10^3\,mL^{-1}$) was used as a model for studying the ability of bacteriocin-producing *Lactobacillus gasseri* K7 (Rif^r) to inhibit clostridia. The added lactobacilli did not inhibit the primary starter culture (*Streptococcus thermophilus*), but inhibited non-starter mesophilic lactobacilli. Late blowing as a result of *Cl. tyrobutyricum* outgrowth and butyric acid fermentation occurred in all cheeses however it was reduced in cheeses with added *Lb. gasseri*. After 6 weeks, the average amount of butyric acid was significantly higher in cheeses without added lactobacilli (1.43 vs. $0.70\,g\,kg^{-1}$). At the end of 8-weeks ripening, 2.8×10^7 cfu g^{-1} of K7 (Rif^r) viable cells were detected. Using the total DNA from cheeses with added K7 (Rif^r) strain, PCR products were amplified with primers specific for *Lactobacillus*, *Lb. gasseri* and K7 bacteriocin gene.

Keywords: *Lactobacillus gasseri*; Bacteriocins; *Clostridium tyrobutyricum*; Cheese; Late blowing

1. Introduction

Clostridium tyrobutyricum strains are often responsible for late blowing of semi-hard and hard cheeses, which is a defect of great economical concern (Bergére & Lenoir, 2000; Klijn, Nieuwenhof, Hoolwerf, Van Der Waals, & Weerkamp, 1995). The most common approaches to prevent this defect include bactofugation or microfiltration of milk, and addition of nitrate or lysozyme (Lodi, 1990; Stadhouders, 1990; van den Berg, Meijer, Düsterhöft, & Smit, 2004). The mechanism of preventing butyric acid fermentation by the use of nitrate is based on the xanthine-oxidase-mediated reduction of nitrate to nitrite, which is able to delay the germination of spores. However, it is also well known that in acidic conditions nitrite can react with aromatic amino acids in cheese to produce potentially carcinogenic nitrosamines (Beresford, Fitzsimons, Brennan, & Cogan, 2001). Therefore nitrate addition, although effective, is not desired because of increasing concern about possible negative effects on health. In addition, the use of nitrate in Emmental, Maasdamer or Gruyère type cheeses have to be limited also since it can inhibit propionic acid fermentation (Bergére & Lenoir, 2000).

Lysozyme, on the other hand, is able to lyse the cell walls of the vegetative form of *Cl. tyrobutyricum* through the enzymatic cleavage and consequently to control clostridial growth and butyric acid fermentation during the maturation of cheeses, in particular those made from pressed and cooked curds, e.g., Swiss Cheese, Parmesan, Edam, Gouda, Cheddar and many others. Although there are some limitations to effective use of lysozyme such us resistance of particular clostridia spores, sensitivity of starter cultures and high number of spores, this approach is suitable for certain types of cheese, when the concentration of clostridial spores is not too high (Lodi, 1990). Lysozyme has approval as a preservative (E1105) in the E.U. Directive on food additives, as well as was affirmed as Generally Recognized as safe (GRAS) for use in cheese by the US Food and Drug Administration, and its use in cheese production is widespread. However, as the residual concentrations of lysozyme in cheese are considerable ($250–400\,mg\,kg^{-1}$) and the well-documented reports on the food allergy are accumulating, some concerns about

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potential allergenicity of lysozyme in egg allergic consumers have arisen recently (Fremont, Kanny, Nicolas, & Moneret-Vautrin, 1997; NDA, 2005).

An alternative to the use of above-mentioned treatments or additives is application of so-called protective lactic acid bacteria (LAB) cultures (Holzapfel, Geisen, & Schillinger, 1995), which can be designated also as functional starter cultures (Leroy & De Vuyst, 2004). The idea of using protective cultures to ensure the additional microbiological safety of food, and also to reduce spoilage organisms, is not new; however, not much information has been available until recently about the use of bacteriocinogenic LAB bacteria for such purposes. In some recent studies the production of bacteriocins (pediocins, lacticins, enterocins and nisin) by LAB in cheese was demonstrated (Bouksaim, Lacroix, Audet, & Simard, 2000; Foulquié Moreno, Rea, Cogan, & De Vuyst, 2003; Giraffa, Picchioni, Neviani, & Carminati, 1995; McAuliffe, Hill, & Ross, 1999; Nuñez, Rodríguez, García, Gaya, & Medina, 1997; Rilla, Martínez, Delgado, & Rodríguez, 2003; Rodríguez, Calzada, Arqués, Rodríguez, Nuñez, & Medina, 2005). In situ produced bacteriocins may increase the competitiveness of producer bacteria, and consequently enable anti-pathogenic activity. The most extensively studied were nisin producing *Lactococcus lactis* strains found to be efficient against non-desirable bacteria such as *Listeria* and *Staphylococcus aureus*, as well as against *Cl. tyrobutyricum* growth in semi-hard cheese (Rilla et al., 2003; Rodríguez et al., 2005). However, the negative effect of bacteriocinogenic strains can be inhibition of not only pathogenic or spoilage bacteria, but also starter bacteria or others which are important in the ripening process, therefore the effect of adding bacteriocinogenic strains to particular food should be carefully examined in laboratory conditions as well as in practice (Leroy & De Vuyst, 2004). Nisin is widely used in cheese and other products as a food additive (Delves-Broughton, 1998), and also approved for use as a bio-preservative in several countries.

Bacteriocins of lactobacilli, less examined in situ in food, have usually a very narrow spectrum of activity. *Lactobacillus sakei* and its bacteriocin sakacin K were tested against *Listeria innocua* in sausages (Leroy & De Vuyst, 2005). More studies were focused on the application of intestinal isolates of lactobacilli as probiotic cultures for cheese (Bergamini, Hynes, Quiberoni, Suarez, & Zalazar, 2005; Gardiner, Ross, Collins, Fitzgerald, & Stanton, 1998; Gardiner, Stanton, Lynch, Collins, Fitzgerald, & Ross, 1999; Gomes, Malcata, Klaver, & Grande, 1995; Kasimoğlu, Göncüoğlu, & Akgün, 2004; Perko, Bogovič Matijašić, & Rogelj, 2002). The prerequisite for the use of particular probiotic strains which most often belong to the *Lb. acidophilus* or *Lb. casei* group in cheese is their compatibility with starter culture organisms, as the latter have to be applied to ensure successful acidification of the curd and the proper ripening process. Usually the required level of probiotic concentration at the moment of intake is established to be at least 10^7 cfu g^{-1} of food. Successful incorporation of probiotic bacteria has been shown in different cheeses produced with mesophilic as well as thermophilic starter cultures (Bergamini et al., 2005).

The human isolate *Lb. gasseri* K7 produces bacteriocins with a wide range of inhibition and has some other probiotic properties such as resistance to low pH and bile, and good survival in vivo, in the pig model as well as in cheese (Bogovič Matijašić & Rogelj, 2000; Čanžek Majhenič, Bogovič Matijašić, & Rogelj, 2003; Perko et al., 2002; Rogelj, Bogovič Matijašić, Čanžek Majhenič, & Stojković, 2002). Its bacteriocins were also found to inhibit several *Cl. tyrobutyricum* vegetative cells and spores in vitro (Bogovič Matijašić & Rogelj, 2000). In the present work, *Lb. gasseri* K7 (Rif^r), a rifampicin-resistant derivative of *Lb. gasseri* K7, was combined with *Streptococcus thermophilus* commercial starter culture to produce a semi-hard cheese. Cheese milk was inoculated with high doses of clostridia spores in order to provoke butyric acid fermentation and consequent blowing of cheeses. In previous reports, thermophilic starter cultures containing *Str. thermophilus* have been combined with bacteriocin-producing lactococci strains (nisin, lacticin 481) (Ávila, Garde, Gaya, Medine, & Nunez, 2005) however, so far no material has been published on testing bacteriocin-producing lactobacilli as protective cultures in cheeses.

Bearing this in mind, we tested the ability of the bacteriocinogenic strain with previously documented probiotic activity and good survival in cheese, to inhibit clostridia and thus contribute positively to the quality and additional nutrition value of cheese (Bogovič Matijašić & Rogelj, 2000; Perko et al., 2002).

2. Materials and methods

2.1. Bacteria and culture conditions

A derivative of *Lb. gasseri* K7 strain, resistant to rifampicin (250 μg mL^{-1}) was obtained by sub-culturing K7 cells in MRS broth (Merck, D-64271 Darmstadt, Germany) with increasing concentration of added rifampicin (Sigma-Aldrich Chemie, D-89552 Steinheim, Germany) and incubation at 37 °C for 18 h. *Cl. tyrobutyricum* 1551 and 1559 isolates from blown cheese were kindly provided by the Federal Dairy Research Institute, Bern, Switzerland.

Eighteen hour *Lb. gasseri* K7 (Rif^r) culture in MRS broth (Merck, 64271 Darmstadt, Germany) with 250 μg mL^{-1} of rifampicin was inoculated (1%) in 1.5 L of the same medium without rifampicin and incubated at 37 °C for 18 h. After centrifugation of the culture (6000 × *g*, 10 min, 4 °C), bacterial cells were washed twice with sterile quarter-strength Ringer solution (Merck, D-64271 Darmstadt, Germany), resuspended in a small amount of milk taken from the cheese vat after thermisation, and transferred into 80 L of milk destined for the cheese manufacture (approximately 10^7 cfu mL^{-1} of milk).

Cl. tyrobutyricum spores were prepared by incubation of RCM broth (Merck, D-64271 Darmstadt, Germany)